ANTISENSE MODULATION OF IL-1 RECEPTOR-ASSOCIATED KINASE-1 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of IL-1 receptor-associated kinase-1. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding IL-1 receptor-associated kinase-1. Such compounds have been shown to modulate the expression of IL-1 receptor-associated kinase-1.

BACKGROUND OF THE INVENTION

The proinflammatory cytokine interleukin-1 (IL-1) is a central regulator in immune and inflammatory responses, involved in generating systemic and local response to infection, injury, and immunologic challenges. IL-1 is produced mainly by activated macrophages and monocytes, and participates in lymphocyte activation, induction of fever, leukocyte trafficking, the acute phase response, and cartilage remodeling. The expression of more than 90 genes is affected by IL-1, including genes that encode other cytokines, cytokine receptors, acute-phase reactants, growth factors, tissue-remodeling enzymes, extracellular matrix components, and cell adhesion molecules. IL-1 is a critical

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cytokine in the pathogenesis of viral infections and
inflammatory diseases such as rheumatoid arthritis (O'Neill
and Greene, *J. Leukoc. Biol.*, **1998**, *63*, 650-657).

5   Cellular responses are transduced through the type I IL-
1 receptor (IL-1RI), located on the plasma membrane of a
variety of IL-1-responsive cells. Binding of IL-1 to IL-1RI
ultimately triggers activation of transcription factors in
the NF-κB family, which are bound by inhibitory proteins
(IκBs) and remain anchored in the cytoplasm until the
10   inhibitory proteins are degraded. In response to IL-1, tumor
necrosis factor (TNF), or other extracellular stimuli such as
lipopolysaccharides, double-stranded RNA, or oxidative
stress, and once unbound and activated, NF-κB is then
transported to the nucleus, where it influences the activity
15   of many genes (O'Neill and Greene, *J. Leukoc. Biol.*, **1998**,
63, 650-657).

          A family of proteins has been described that share
significant homology with the type I IL-1 receptor in their
signaling domains. This family includes IL-1 receptor
20   accessory protein (IL1-RAcP), which does not bind IL-1, but
is essential for IL-1 signaling; a Drosophila receptor
protein, Toll; a number of human Toll-like receptors (hTLRs);
the interferon-γ-inducing factor/IL-1γ/ IL-18 receptor-related
protein (IL-1Rrp), a number of plant proteins, and the IL-1
25   receptor-associated kinases (IRAK-1, IRAK-2, and IRAK-M). All
members of this family appear to be involved in host
responses to injury and infection (O'Neill and Greene, *J.
Leukoc. Biol.*, **1998**, *63*, 650-657; Wesche et al., *J. Biol.
Chem.*, **1999**, *274*, 19403-19410).

30   IL-1 receptor-associated kinase-1 (also known as
interleukin 1 receptor-associated kinase 1, IRAK1, IRAK-1,
Il1rak, mouse Pelle -like kinase, and mPLK) was purified from
human embryonic kidney epithelial cell line 293, and its

amino acid sequence was determined by micropeptide
sequencing. PCR was then used to amplify a probe, and the
gene was cloned from a human teratocarcinoma cDNA library.
The IL-1 receptor-associated kinase-1 gene encodes a 3.5-kb
5   mRNA that was detected in all tissues examined (Cao et al.,
Science, **1996,** *271,* 1128-1131). The IL-1 receptor-associated
kinase-1 gene has been mapped to the human X chromosome
region q28, and a homologous region XA7-C on mouse chromosome
X (Reichwald et al., *Mamm. Genome,* **2000,** *11,* 182-190).

10   The IL-1 signaling pathway in mammals is analogous to
the Toll pathway in *Drosophila melanogaster.* Homologues of
IL-1 receptor-associated kinase-1 are found in *D.*
melanogaster (Pelle) and in plants (Pto), and in these
systems, the kinases have been shown to be components of a
15   signal transduction system leading to the activation of NF-
κB. In fact, because of their homology to several components
of the NF-κB signaling pathway in *D. melanogaster,* new
mammalian proteins of the IL-1RI signaling system and their
functions are being elucidated (Burns et al., *Nat. Cell*
20   *Biol.,* **2000,** *2,* 346-351; O'Neill and Greene, *J. Leukoc.*
Biol., **1998,** *63,* 650-657; Vig et al., *J. Biol. Chem.,* **2001,**
276, 7859-7866). A novel signaling molecule that associates
with the mouse Pelle-like kinase, SIMPL, was recently
identified in mice, and found to bind to IL-1 receptor
25   associated kinase (Vig et al., *J. Biol. Chem.,* **2001,** *276,*
7859-7866). Additionally, a novel protein, Tollip, was found
to associate with the activated IL-1RI/IL-1RAcP complex
(Burns et al., *Nat. Cell Biol.,* **2000,** *2,* 346-351).

          When cells receive the extracellular IL-1 signal, a
30   complex between IL-1RI and IL-1RAcP is formed (Huang et al.,
Proc. Natl. Acad. Sci. U. S. A., **1997,** *94,* 12829-12832), the
cytosolic adapter protein MyD88 interacts with IL-1RAcP in
the receptor complex (Burns et al., *J. Biol. Chem.,* **1998,**

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273, 12203-12209), and MyD88 rapidly recruits IL-1 receptor-
associated kinase-1 into the complex. Tollip also interacts
with IL-1RAcP and is believed to block autophosphorylation of
the IL-1 receptor-associated kinase-1 or its association with
5   another kinase; thus, the association of Tollip with IL-1
receptor-associated kinase-1 is inhibitory (Burns et al.,
Nat. Cell Biol., **2000**, *2*, 346-351). At some point after its
IL-1-dependent association with the receptor complex, IL-1
receptor-associated kinase-1 is extensively phosphorylated
10   and its own serine/threonine kinase catalytic activity
becomes activated (Cao et al., *Science,* **1996**, *271*, 1128-
1131). IL-1 receptor-associated kinase-1 then interacts with
an adapter protein, TRAF6, a protein critical for IL-1-
dependent activation of NF-κB, which dissociates from the
15   receptor complex. TRAF6 relays a signal via NF-κB-inducing
kinase (NIK) to two I-κB kinases (IKK-1 and -2), culminating
in activation of NF-κB (Bacher et al., *FEBS Lett.,* **2001**, *497*,
153-158; Jefferies et al., *Mol. Cell. Biol.,* **2001**, *21*, 4544-
4552; O'Neill and Greene, *J. Leukoc. Biol.,* **1998**, *63*, 650-
20   657).

        Cellular trafficking and nuclear importation may play a
role in the timing and/or activity of IL-1 receptor-
associated kinase-1 mediated signaling. Association of IL-1
receptor-associated kinase-1 with the receptor complex is
25   detectable 30 seconds after IL-1 stimulation of human
umbilical cord vein ECV 304 cells, and significant levels of
IL-1 receptor-associated kinase-1 accumulate in the nucleus
within 30 minutes (Bol et al., *FEBS Lett.,* **2000**, *477*, 73-78).
        There is evidence that hyperphosphorylation of IL-1
30   receptor-associated kinase-1 is regulatory and results in
proteolytic degradation. A nonspecific kinase inhibitor
blocks proteolysis of IL-1 receptor-associated kinase-1 as
well as its phosphorylation, and the translocation of IL-1

receptor-associated kinase-1 to the IL-1RI/IL-1RAcP complex
is independent of this treatment. Degradation of the IL-1
receptor-associated kinase-1 component of this signaling
pathway may explain why some IL-1 responses are transient

5   (Yamin and Miller, *J. Biol. Chem.*, **1997**, *272*, 21540-21547).

IL-1 receptor-associated kinase-1 plays a role in
regulation of multiple signaling pathways. In addition to
transducing the IL-1 signal, IL-1 receptor-associated kinase-
1 also transduces a signal initiated by binding of tumor

10   necrosis factor (TNF)-α. to its receptor, again leading to
activation of NF-κB (Vig et al., *J. Biol. Chem.*, **1999**, *274*,
13077-13084; Vig et al., *J. Biol. Chem.*, **2001**, *276*, 7859-
7866). In another signal transduction pathway separate from
its activation of NF-κB, IL-1 receptor-associated kinase-1

15   has also been implicated in activation of the Jun amino-
terminal kinase (JNK) and the transcription factor AP-1
(Bacher et al., *FEBS Lett.*, **2001**, *497*, 153-158).

The IL-1 receptor-associated kinase-1 gene has been
disrupted in mice. Upon IL-1 treatment, IL-1 receptor-

20   associated kinase-1-deficient embryonic fibroblasts derived
from these mice are defective in activation of JNK, p38 MAP
kinase, and NF-κB (Kanakaraj et al., *J. Exp. Med.*, **1998**, *187*,
2073-2079). Furthermore, in T helper cell type 1 (Th1) cells
from the IL-1 receptor-associated kinase-1 null mice, IL-18-

25   induced production of interferon (IFN)-γ was substantially
reduced, proliferation of the Th1 cells was decreased,
activation of natural killer (NK) cells was defective, and
these defects resulted in an impaired immune response to
murine cytomegalovirus (MCMV) infection (Kanakaraj et al., *J.*

30   *Exp. Med.*, **1999**, *189*, 1129-1138).

In a separate study, IL-1 receptor-associated kinase-1-
deficient mice and fibroblasts were generated, and it was
further demonstrated that IL-1 receptor-associated kinase-1

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null mice retain a normal response to *Listeria monocytogenes* infection (Thomas et al., *J. Immunol.*, **1999**, *163*, 978-984).

The pharmacological modulation of IL-1 receptor-associated kinase-1 activity and/or expression is therefore
5   believed to be an appropriate point of therapeutic intervention in pathological conditions such as viral infections, rheumatoid arthritis, and other inflammatory disease and immune disorders.

To date, investigative strategies aimed at studying the
10 localization and function of IL-1 receptor-associated kinase-1 have involved the use of antibodies, transgenic animals and IL-1 receptor-associated kinase-1-deficient cell lines, and an antisense oligonucleotide.

Disclosed and claimed in US Patent 5,654,397 are nucleic
15 acids which encode IL-1 receptor-associated kinase-1 and methods for screening chemical libraries and identifying lead compounds to be used as pharmacological agents in the diagnosis and treatment of disease associated with interleukin-1 signal transduction (Cao et al., **1997**).

20   IL-1 receptor-associated kinase-1 specific antibodies have been raised and used to study IL-1 receptor-associated kinase-1 (Cao et al., *Science,* **1996**, *271*, 1128-1131) and its interactions with the IL-1RI/IL-1RAcP complex (Volpe et al., *FEBS Lett.,* **1997**, *419*, 41-44) and to demonstrate that the
25 phosphorylated form of IL-1 receptor-associated kinase-1 is degraded (Yamin and Miller, *J. Biol. Chem.,* **1997**, *272*, 21540-21547).

A phosphorothioate antisense oligonucleotide, 18 nucleotides in length, targeted to a region from nucleotide -
30 6 to nucleotide 12 of the mRNA encoding the human IL-1 receptor-associated kinase-1 and spanning the translation initiation site, was used to show that inhibition of expression of IL-1 receptor-associated kinase-1 blocks

activation of NF-κB (Guo and Wu, *Immunopharmacology*, **2000**, *49*, 241-246).

Disclosed and claimed in US Patent 6,166,289 is a transgenic mouse whose somatic and germ cells comprise a

5   disruption in an endogenous IL-1 receptor-associated kinase-1 gene, wherein the disruption is generated by targeted replacement with a non-functional IL-1 receptor-associated kinase-1 gene, and wherein said disruption results in IL-1 receptor-associated kinase-1-deficient cells from said mouse

10 having a decrease in activation of JNK, activation of p38, and induction of IL-6 in response to IL-1 as compared to wild-type mice, as well as a method for producing this transgenic mouse (Harris et al., **2000**).

Disclosed and claimed in US Patent 6,127,176 is a mutant

15 cell that lacks a functional IL-1 receptor-associated kinase-1 and comprise an HSV thymidine kinase gene operatively linked to an IL-1 promoter and zeomycin resistance gene operatively linked to an IL-1 promoter, as well as a method of making a mutant mammalian cell that lacks a functional

20 component of the IL-1 signaling pathway and the TNF signaling pathways (Stark and Li, **2000**).

Disclosed and claimed in US Patent 5,817,479 are polynucleotides which identify and encode novel protein kinases expressed in various human cells and tissues, wherein

25 one of the kinases is IL-1 receptor-associated kinase-1. Further claimed are expression vectors, host cells, and methods for the production of purified kinase peptides, antibodies capable of binding the kinases, and inhibitors of the kinases as well as antisense sequences and

30 oligonucleotides designed from the polynucleotides or their complements (Au-Young et al., **1998**).

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of IL-1 receptor-associated

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kinase-1. Consequently, there remains a long felt need for
agents capable of effectively inhibiting IL-1 receptor-
associated kinase-1 function.

Antisense technology is emerging as an effective means
for reducing the expression of specific gene products and may
therefore prove to be uniquely useful in a number of
therapeutic, diagnostic, and research applications for the
modulation of IL-1 receptor-associated kinase-1 expression.

The present invention provides compositions and methods
for modulating IL-1 receptor-associated kinase-1 expression.

SUMMARY OF THE INVENTION

The present invention is directed to compounds,
particularly antisense oligonucleotides, which are targeted
to a nucleic acid encoding IL-1 receptor-associated kinase-1,
and which modulate the expression of IL-1 receptor-associated
kinase-1. Pharmaceutical and other compositions comprising
the compounds of the invention are also provided. Further
provided are methods of modulating the expression of IL-1
receptor-associated kinase-1 in cells or tissues comprising
contacting said cells or tissues with one or more of the
antisense compounds or compositions of the invention.
Further provided are methods of treating an animal,
particularly a human, suspected of having or being prone to a
disease or condition associated with expression of IL-1
receptor-associated kinase-1 by administering a
therapeutically or prophylactically effective amount of one
or more of the antisense compounds or compositions of the
invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds,

particularly antisense oligonucleotides, for use in
modulating the function of nucleic acid molecules encoding
IL-1 receptor-associated kinase-1, ultimately modulating the
amount of IL-1 receptor-associated kinase-1 produced. This
5   is accomplished by providing antisense compounds which
specifically hybridize with one or more nucleic acids
encoding IL-1 receptor-associated kinase-1. As used herein,
the terms "target nucleic acid" and "nucleic acid encoding
IL-1 receptor-associated kinase-1" encompass DNA encoding IL-
10 1 receptor-associated kinase-1, RNA (including pre-mRNA and
mRNA) transcribed from such DNA, and also cDNA derived from
such RNA. The specific hybridization of an oligomeric
compound with its target nucleic acid interferes with the
normal function of the nucleic acid. This modulation of
15 function of a target nucleic acid by compounds which
specifically hybridize to it is generally referred to as
"antisense". The functions of DNA to be interfered with
include replication and transcription. The functions of RNA
to be interfered with include all vital functions such as,
20 for example, translocation of the RNA to the site of protein
translation, translocation of the RNA to sites within the
cell which are distant from the site of RNA synthesis,
translation of protein from the RNA, splicing of the RNA to
yield one or more mRNA species, and catalytic activity which
25 may be engaged in or facilitated by the RNA. The overall
effect of such interference with target nucleic acid function
is modulation of the expression of IL-1 receptor-associated
kinase-1. In the context of the present invention,
"modulation" means either an increase (stimulation) or a
30 decrease (inhibition) in the expression of a gene. In the
context of the present invention, inhibition is the preferred
form of modulation of gene expression and mRNA is a preferred
target.

It is preferred to target specific nucleic acids for
antisense. "Targeting" an antisense compound to a particular
nucleic acid, in the context of this invention, is a
multistep process. The process usually begins with the
5 identification of a nucleic acid sequence whose function is
to be modulated. This may be, for example, a cellular gene
(or mRNA transcribed from the gene) whose expression is
associated with a particular disorder or disease state, or a
nucleic acid molecule from an infectious agent. In the
10 present invention, the target is a nucleic acid molecule
encoding IL-1 receptor-associated kinase-1. The targeting
process also includes determination of a site or sites within
this gene for the antisense interaction to occur such that
the desired effect, e.g., detection or modulation of
15 expression of the protein, will result. Within the context
of the present invention, a preferred intragenic site is the
region encompassing the translation initiation or termination
codon of the open reading frame (ORF) of the gene. Since, as
is known in the art, the translation initiation codon is
20 typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in
the corresponding DNA molecule), the translation initiation
codon is also referred to as the "AUG codon," the "start
codon" or the "AUG start codon". A minority of genes have a
translation initiation codon having the RNA sequence 5'-GUG,
25 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been
shown to function *in vivo*. Thus, the terms "translation
initiation codon" and "start codon" can encompass many codon
sequences, even though the initiator amino acid in each
instance is typically methionine (in eukaryotes) or
30 formylmethionine (in prokaryotes). It is also known in the
art that eukaryotic and prokaryotic genes may have two or
more alternative start codons, any one of which may be
preferentially utilized for translation initiation in a

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particular cell type or tissue, or under a particular set of
conditions. In the context of the invention, "start codon"
and "translation initiation codon" refer to the codon or
codons that are used *in vivo* to initiate translation of an
5   mRNA molecule transcribed from a gene encoding IL-1 receptor-
associated kinase-1, regardless of the sequence(s) of such
codons.

It is also known in the art that a translation
termination codon (or "stop codon") of a gene may have one of
10   three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the
corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA,
respectively). The terms "start codon region" and
"translation initiation codon region" refer to a portion of
such an mRNA or gene that encompasses from about 25 to about
15   50 contiguous nucleotides in either direction (i.e., 5' or
3') from a translation initiation codon. Similarly, the
terms "stop codon region" and "translation termination codon
 region" refer to a portion of such an mRNA or gene that
encompasses from about 25 to about 50 contiguous nucleotides
20   in either direction (i.e., 5' or 3') from a translation
termination codon.

The open reading frame (ORF) or "coding region," which
is known in the art to refer to the region between the
translation initiation codon and the translation termination
25   codon, is also a region which may be targeted effectively.
Other target regions include the 5' untranslated region
(5'UTR), known in the art to refer to the portion of an mRNA
in the 5' direction from the translation initiation codon,
and thus including nucleotides between the 5' cap site and
30   the translation initiation codon of an mRNA or corresponding
nucleotides on the gene, and the 3' untranslated region
(3'UTR), known in the art to refer to the portion of an mRNA
in the 3' direction from the translation termination codon,

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and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue

5 of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

10   Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a

15 continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease.

20 Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It has also been found that introns can be effective, and therefore

25 preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

      It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as

30 "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in

either their start or stop position and contain both intronic and extronic regions.

Upon excision of one or more exon or intron regions or portions thereof during splicing, pre-mRNA variants produce
5 smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant
10 occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess
15 more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-
20 mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.
25 Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.
30 In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and

thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain

5   position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are

10 complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of

15 complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be

20 specifically hybridizable.

An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of activity, and there is a sufficient degree

25 of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays,

30 under conditions in which the assays are performed. It is preferred that the antisense compounds of the present invention comprise at least 80% sequence complementarity to a target region within the target nucleic acid, moreover that

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they comprise 90% sequence complementarity and even more comprise 95% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense compound in which 18 of
5 20 nucleobases of the antisense compound are complementary, and would therefore specifically hybridize, to a target region would represent 90 percent complementarity. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using basic
10 local alignment search tools (BLAST programs) (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target,
15 are identified through experimentation, and representative sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The sites to which these preferred antisense compounds are specifically hybridizable are hereinbelow referred to as "preferred target
20 regions" and are therefore preferred sites for targeting. As used herein the term "preferred target region" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these
25 target regions represent regions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of particular preferred target regions are set forth below, one of skill in the art will recognize that these serve to illustrate and describe
30 particular embodiments within the scope of the present invention. Additional preferred target regions may be identified by one having ordinary skill.

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Target regions 8-80 nucleobases in length comprising a
stretch of at least eight (8) consecutive nucleobases
selected from within the illustrative preferred target
regions are considered to be suitable preferred target
5   regions as well.

Exemplary good preferred target regions include DNA or
RNA sequences that comprise at least the 8 consecutive
nucleobases from the 5'-terminus of one of the illustrative
preferred target regions (the remaining nucleobases being a
10 consecutive stretch of the same DNA or RNA beginning
immediately upstream of the 5'-terminus of the target region
and continuing until the DNA or RNA contains about 8 to about
80 nucleobases). Similarly good preferred target regions are
represented by DNA or RNA sequences that comprise at least
15 the 8 consecutive nucleobases from the 3'-terminus of one of
the illustrative preferred target regions (the remaining
nucleobases being a consecutive stretch of the same DNA or
RNA beginning immediately downstream of the 3'-terminus of
the target region and continuing until the DNA or RNA
20 contains about 8 to about 80 nucleobases). One having skill
in the art, once armed with the empirically-derived preferred
target regions illustrated herein will be able, without undue
experimentation, to identify further preferred target
regions. In addition, one having ordinary skill in the art
25 will also be able to identify additional compounds, including
oligonucleotide probes and primers, that specifically
hybridize to these preferred target regions using techniques
available to the ordinary practitioner in the art.

Antisense compounds are commonly used as research
30 reagents and diagnostics. For example, antisense
oligonucleotides, which are able to inhibit gene expression
with exquisite specificity, are often used by those of
ordinary skill to elucidate the function of particular genes.

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Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

5   For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire

10   complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of

15   gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other

20   compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene

25   expression)(Madden, *et al.*, *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81),

30   protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, *et al.*, *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000**, 480, 2-16; Larsson, *et al.*, *J.*

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Biotechnol., **2000**, 80, 143-57), subtractive RNA
fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000**,
286, 91-98; Larson, *et al.*, *Cytometry*, **2000**, 41, 203-208),
subtractive cloning, differential display (DD) (Jurecic and
5   Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21),
comparative genomic hybridization (Carulli, *et al.*, *J. Cell
Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent *in situ*
hybridization) techniques (Going and Gusterson, *Eur. J.
Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods
10   (reviewed in To, *Comb. Chem. High Throughput Screen*, **2000**, 3,
235-41).

The specificity and sensitivity of antisense is also
harnessed by those of skill in the art for therapeutic uses.
Antisense oligonucleotides have been employed as therapeutic
15   moieties in the treatment of disease states in animals and
man. Antisense oligonucleotide drugs, including ribozymes,
have been safely and effectively administered to humans and
numerous clinical trials are presently underway. It is thus
established that oligonucleotides can be useful therapeutic
20   modalities that can be configured to be useful in treatment
regimes for treatment of cells, tissues and animals,
especially humans.

In the context of this invention, the term
"oligonucleotide" refers to an oligomer or polymer of
25   ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or
mimetics thereof. This term includes oligonucleotides
composed of naturally-occurring nucleobases, sugars and
covalent internucleoside (backbone) linkages as well as
oligonucleotides having non-naturally-occurring portions
30   which function similarly. Such modified or substituted
oligonucleotides are often preferred over native forms
because of desirable properties such as, for example,
enhanced cellular uptake, enhanced affinity for nucleic acid

target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides from about 8 to about 50 nucleobases, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

Antisense compounds 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative antisense compounds are considered to be suitable antisense compounds as well.

Exemplary preferred antisense compounds include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred antisense compounds are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense

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compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and

5 continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense

10 compounds.

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are herein identified as

15 preferred embodiments of the invention. While specific sequences of the antisense compounds are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred

20 antisense compounds may be identified by one having ordinary skill.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such

25 heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to

30 either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this

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linear polymeric structure can be further joined to form a
circular structure, however, open linear structures are
generally preferred. In addition, linear structures may also
have internal nucleobase complementarity and may therefore
fold in a manner as to produce a double stranded structure.
Within the oligonucleotide structure, the phosphate groups
are commonly referred to as forming the internucleoside
backbone of the oligonucleotide. The normal linkage or
backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds
useful in this invention include oligonucleotides containing
modified backbones or non-natural internucleoside linkages.
As defined in this specification, oligonucleotides having
modified backbones include those that retain a phosphorus
atom in the backbone and those that do not have a phosphorus
atom in the backbone. For the purposes of this
specification, and as sometimes referenced in the art,
modified oligonucleotides that do not have a phosphorus atom
in their internucleoside backbone can also be considered to
be oligonucleosides.

Preferred modified oligonucleotide backbones include,
for example, phosphorothioates, chiral phosphorothioates,
phosphorodithioates, phosphotriesters, aminoalkylphosphotri-
esters, methyl and other alkyl phosphonates including 3'-
alkylene phosphonates, 5'-alkylene phosphonates and chiral
phosphonates, phosphinates, phosphoramidates including 3'-
amino phosphoramidate and aminoalkylphosphoramidates,
thionophosphoramidates, thionoalkylphosphonates,
thionoalkylphosphotriesters, selenophosphates and borano-
phosphates having normal 3'-5' linkages, 2'-5' linked analogs
of these, and those having inverted polarity wherein one or
more internucleotide linkages is a 3' to 3', 5' to 5' or 2'
to 2' linkage. Preferred oligonucleotides having inverted

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polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed

5   salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423;

10   5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are

15   commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside

20   linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones;

25   sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide

30   backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are

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not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444;
5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564;
5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677;
5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;
5   5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;
5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and
5,677,439, certain of which are commonly owned with this
application, and each of which is herein incorporated by
reference.

10   In other preferred oligonucleotide mimetics, both the
sugar and the internucleoside linkage, i.e., the backbone, of
the nucleotide units are replaced with novel groups. The
base units are maintained for hybridization with an
appropriate nucleic acid target compound. One such
15 oligomeric compound, an oligonucleotide mimetic that has been
shown to have excellent hybridization properties, is referred
to as a peptide nucleic acid (PNA). In PNA compounds, the
sugar-backbone of an oligonucleotide is replaced with an
amide containing backbone, in particular an aminoethylglycine
20 backbone. The nucleobases are retained and are bound
directly or indirectly to aza nitrogen atoms of the amide
portion of the backbone. Representative United States
patents that teach the preparation of PNA compounds include,
but are not limited to, U.S.: 5,539,082; 5,714,331; and
25 5,719,262, each of which is herein incorporated by reference.
Further teaching of PNA compounds can be found in Nielsen *et
al.*, *Science*, **1991**, 254, 1497-1500.

     Most preferred embodiments of the invention are
oligonucleotides with phosphorothioate backbones and
30 oligonucleosides with heteroatom backbones, and in particular
$-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene
(methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-$
$N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native

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phosphodiester backbone is represented as -O-P-O-CH$_2$-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C$_1$ to C$_{10}$ alkyl or C$_2$ to C$_{10}$ alkenyl and alkynyl. Particularly preferred are O[(CH$_2$)$_n$O]$_m$CH$_3$, O(CH$_2$)$_n$OCH$_3$, O(CH$_2$)$_n$NH$_2$, O(CH$_2$)$_n$CH$_3$, O(CH$_2$)$_n$ONH$_2$, and O(CH$_2$)$_n$ON[(CH$_2$)$_n$CH$_3$]$_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C$_1$ to C$_{10}$ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH$_3$, OCN, Cl, Br, CN, CF$_3$, OCF$_3$, SOCH$_3$, SO$_2$CH$_3$, ONO$_2$, NO$_2$, N$_3$, NH$_2$, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH$_2$CH$_2$OCH$_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a O(CH$_2$)$_2$ON(CH$_3$)$_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-

dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH$_2$-O-CH$_2$-N(CH$_3$)$_2$, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH$_3$), 2'-aminopropoxy (2'-OCH$_2$CH$_2$CH$_2$NH$_2$), 2'-allyl (2'-CH$_2$-

5   CH=CH$_2$), 2'-O-allyl (2'-O-CH$_2$-CH=CH$_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3'

10   position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the

15   preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873;

20   5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the

25   3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne (-CH$_2$-)$_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

30   Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine

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(G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et *al.*, *Angewandte Chemie*,

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International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are

5   particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine

10   substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when

15   combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as

20   well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned

25   with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the

30   invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include

conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers,

5 groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluores-

10 ceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that

15 enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire

20 disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-

25 1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or

30 undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov *et al.*, *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk *et al.*, *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-

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ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate
(Manoharan *et al.*, *Tetrahedron Lett.*, **1995**, *36*, 3651-3654;
Shea *et al.*, *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a
polyamine or a polyethylene glycol chain (Manoharan *et al.*,

5   *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane
acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, **1995**, *36*,
3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim.*
Biophys. Acta, **1995**, *1264*, 229-237), or an octadecylamine or
hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J.*

10   *Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937). Oligonucleotides
of the invention may also be conjugated to active drug
substances, for example, aspirin, warfarin, phenylbutazone,
ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-
pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic

15   acid, flufenamic acid, folinic acid, a benzothiadiazide,
chlorothiazide, a diazepine, indomethicin, a barbiturate, a
cephalosporin, a sulfa drug, an antidiabetic, an
antibacterial or an antibiotic. Oligonucleotide-drug
conjugates and their preparation are described in United

20   States Patent Application 09/334,130 (filed June 15, 1999)
which is incorporated herein by reference in its entirety.

Representative United States patents that teach the
preparation of such oligonucleotide conjugates include, but
are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105;

25   5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717,
5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;
5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;
5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;

30   4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;
5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,
5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;

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5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNAse H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as interferon-induced RNAseL which cleaves both cellular and viral RNA. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric

oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid

5 hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such

10 compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065;

15 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the

20 well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar

25 techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as

30 for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such

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uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556;

5   5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

10   The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite

15   or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

         The term "prodrug" indicates a therapeutic agent that is

20   prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE

25   [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

         The term "pharmaceutically acceptable salts" refers to

30   physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

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Pharmaceutically acceptable base addition salts are
formed with metals or amines, such as alkali and alkaline
earth metals or organic amines. Examples of metals used as
cations are sodium, potassium, magnesium, calcium, and the
like. Examples of suitable amines are
N,N'-dibenzylethylenediamine, chloroprocaine, choline,
diethanolamine, dicyclohexylamine, ethylenediamine,
N-methylglucamine, and procaine (see, for example, Berge et
al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-
19). The base addition salts of said acidic compounds are
prepared by contacting the free acid form with a sufficient
amount of the desired base to produce the salt in the
conventional manner. The free acid form may be regenerated
by contacting the salt form with an acid and isolating the
free acid in the conventional manner. The free acid forms
differ from their respective salt forms somewhat in certain
physical properties such as solubility in polar solvents, but
otherwise the salts are equivalent to their respective free
acid for purposes of the present invention. As used herein,
a "pharmaceutical addition salt" includes a pharmaceutically
acceptable salt of an acid form of one of the components of
the compositions of the invention. These include organic or
inorganic acid salts of the amines. Preferred acid salts are
the hydrochlorides, acetates, salicylates, nitrates and
phosphates. Other suitable pharmaceutically acceptable salts
are well known to those skilled in the art and include basic
salts of a variety of inorganic and organic acids, such as,
for example, with inorganic acids, such as for example
hydrochloric acid, hydrobromic acid, sulfuric acid or
phosphoric acid; with organic carboxylic, sulfonic, sulfo or
phospho acids or N-substituted sulfamic acids, for example
acetic acid, propionic acid, glycolic acid, succinic acid,
maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric

acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid,

5   2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic

10   acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates),

15   or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth,

20   ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

       For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium,

25   ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for

30   example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid,

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naphthalenesulfonic acid, methanesulfonic acid,
p-toluenesulfonic acid, naphthalenedisulfonic acid,
polygalacturonic acid, and the like; and (d) salts formed
from elemental anions such as chlorine, bromine, and iodine.

5   The antisense compounds of the present invention can be
utilized for diagnostics, therapeutics, prophylaxis and as
research reagents and kits. For therapeutics, an animal,
preferably a human, suspected of having a disease or disorder
which can be treated by modulating the expression of IL-1

10   receptor-associated kinase-1 is treated by administering
antisense compounds in accordance with this invention. The
compounds of the invention can be utilized in pharmaceutical
compositions by adding an effective amount of an antisense
compound to a suitable pharmaceutically acceptable diluent or

15   carrier. Use of the antisense compounds and methods of the
invention may also be useful prophylactically, e.g., to
prevent or delay infection, inflammation or tumor formation,
for example.

          The antisense compounds of the invention are useful for

20   research and diagnostics, because these compounds hybridize
to nucleic acids encoding IL-1 receptor-associated kinase-1,
enabling sandwich and other assays to easily be constructed
to exploit this fact. Hybridization of the antisense
oligonucleotides of the invention with a nucleic acid

25   encoding IL-1 receptor-associated kinase-1 can be detected by
means known in the art. Such means may include conjugation
of an enzyme to the oligonucleotide, radiolabelling of the
oligonucleotide or any other suitable detection means. Kits
using such detection means for detecting the level of IL-1

30   receptor-associated kinase-1 in a sample may also be
prepared.

          The present invention also includes pharmaceutical
compositions and formulations which include the antisense

compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may
5 be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration
10 includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly
15 useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous,
20 powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids,
25 liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g.
30 dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes

thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic

5 acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g.

10 isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

15 Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents,

20 emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include

25 fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid,

30 taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid,

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palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a

5   pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further

10   penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include

15   poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines,

20   pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino),

25   poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-

30   lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July

1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999), each of which is incorporated herein by reference in their entirety.

5   Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and

10   other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are

15   not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques

20   well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active

25   ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as,

30   but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media.

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Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

5   In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature

10   these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

15

Emulsions

          The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in

20   another in the form of droplets usually exceeding 0.1 µm in diameter (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**,

25   Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301).

30   Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When

an aqueous phase is finely divided into and dispersed as
minute droplets into a bulk oily phase, the resulting
composition is called a water-in-oil (w/o) emulsion.
Alternatively, when an oily phase is finely divided into and
5   dispersed as minute droplets into a bulk aqueous phase, the
resulting composition is called an oil-in-water (o/w)
emulsion. Emulsions may contain additional components in
addition to the dispersed phases, and the active drug which
may be present as a solution in either the aqueous phase,
10   oily phase or itself as a separate phase. Pharmaceutical
excipients such as emulsifiers, stabilizers, dyes, and anti-
oxidants may also be present in emulsions as needed.
Pharmaceutical emulsions may also be multiple emulsions that
are comprised of more than two phases such as, for example,
15   in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-
in-water (w/o/w) emulsions. Such complex formulations often
provide certain advantages that simple binary emulsions do
not. Multiple emulsions in which individual oil droplets of
an o/w emulsion enclose small water droplets constitute a
20   w/o/w emulsion. Likewise a system of oil droplets enclosed
in globules of water stabilized in an oily continuous phase
provides an o/w/o emulsion.

Emulsions are characterized by little or no
thermodynamic stability. Often, the dispersed or
25   discontinuous phase of the emulsion is well dispersed into
the external or continuous phase and maintained in this form
through the means of emulsifiers or the viscosity of the
formulation. Either of the phases of the emulsion may be a
semisolid or a solid, as is the case of emulsion-style
30   ointment bases and creams. Other means of stabilizing
emulsions entail the use of emulsifiers that may be
incorporated into either phase of the emulsion. Emulsifiers
may broadly be classified into four categories: synthetic

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surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

5   Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1,

10   p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic

15   nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group:

20   nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

         Naturally occurring emulsifiers used in emulsion

25   formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids

30   have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite,

hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also
5   included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker
10 (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally
15 occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose
20 ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

25   Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include
30 methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation.

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Antioxidants used may be free radical scavengers such as
tocopherols, alkyl gallates, butylated hydroxyanisole,
butylated hydroxytoluene, or reducing agents such as ascorbic
acid and sodium metabisulfite, and antioxidant synergists
5   such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via
dermatological, oral and parenteral routes and methods for
their manufacture have been reviewed in the literature
(Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and
10 Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y.,
volume 1, p. 199). Emulsion formulations for oral delivery
have been very widely used because of ease of formulation, as
well as efficacy from an absorption and bioavailability
standpoint (Rosoff, in *Pharmaceutical Dosage Forms*,
15 Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker,
Inc., New York, N.Y., volume 1, p. 245; Idson, in
Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker
(Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1,
p. 199). Mineral-oil base laxatives, oil-soluble vitamins
20 and high fat nutritive preparations are among the materials
that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the
compositions of oligonucleotides and nucleic acids are
formulated as microemulsions. A microemulsion may be defined
25 as a system of water, oil and amphiphile which is a single
optically isotropic and thermodynamically stable liquid
solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman,
Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New
York, N.Y., volume 1, p. 245). Typically microemulsions are
30 systems that are prepared by first dispersing an oil in an
aqueous surfactant solution and then adding a sufficient
amount of a fourth component, generally an intermediate
chain-length alcohol to form a transparent system.

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Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in:

5 *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the

10 microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*,

15 Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*,

20 Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions

25 offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic

30 surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500),

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decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain

5   alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without

10   the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene

15   glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated

20   polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have

25   been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., Pharmaceutical Research, **1994**, *11*, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of

30   drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical

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potency, and decreased toxicity (Constantinides et al.,
Pharmaceutical Research, **1994**, *11*, 1385; Ho et al., *J. Pharm.
Sci.*, **1996**, *85*, 138-143). Often microemulsions may form
spontaneously when their components are brought together at
5   ambient temperature. This may be particularly advantageous
when formulating thermolabile drugs, peptides or
oligonucleotides. Microemulsions have also been effective in
the transdermal delivery of active components in both
cosmetic and pharmaceutical applications. It is expected
10 that the microemulsion compositions and formulations of the
present invention will facilitate the increased systemic
absorption of oligonucleotides and nucleic acids from the
gastrointestinal tract, as well as improve the local cellular
uptake of oligonucleotides and nucleic acids within the
15 gastrointestinal tract, vagina, buccal cavity and other areas
of administration.

   Microemulsions of the present invention may also contain
additional components and additives such as sorbitan
monostearate (Grill 3), Labrasol, and penetration enhancers
20 to improve the properties of the formulation and to enhance
the absorption of the oligonucleotides and nucleic acids of
the present invention. Penetration enhancers used in the
microemulsions of the present invention may be classified as
belonging to one of five broad categories - surfactants,
25 fatty acids, bile salts, chelating agents, and non-chelating
non-surfactants (Lee et al., *Critical Reviews in Therapeutic
Drug Carrier Systems*, **1991**, p. 92). Each of these classes
has been discussed above.

30 Liposomes
   There are many organized surfactant structures besides
microemulsions that have been studied and used for the
formulation of drugs. These include monolayers, micelles,

bilayers and vesicles. Vesicles, such as liposomes, have
attracted great interest because of their specificity and the
duration of action they offer from the standpoint of drug
delivery. As used in the present invention, the term
5   "liposome" means a vesicle composed of amphiphilic lipids
arranged in a spherical bilayer or bilayers.

     Liposomes are unilamellar or multilamellar vesicles
which have a membrane formed from a lipophilic material and
an aqueous interior. The aqueous portion contains the
10 composition to be delivered. Cationic liposomes possess the
advantage of being able to fuse to the cell wall. Non-
cationic liposomes, although not able to fuse as efficiently
with the cell wall, are taken up by macrophages *in vivo*.

     In order to cross intact mammalian skin, lipid vesicles
15 must pass through a series of fine pores, each with a
diameter less than 50 nm, under the influence of a suitable
transdermal gradient. Therefore, it is desirable to use a
liposome which is highly deformable and able to pass through
such fine pores.

20   Further advantages of liposomes include; liposomes
obtained from natural phospholipids are biocompatible and
biodegradable; liposomes can incorporate a wide range of
water and lipid soluble drugs; liposomes can protect
encapsulated drugs in their internal compartments from
25 metabolism and degradation (Rosoff, in *Pharmaceutical Dosage
Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel
Dekker, Inc., New York, N.Y., volume 1, p. 245). Important
considerations in the preparation of liposome formulations
are the lipid surface charge, vesicle size and the aqueous
30 volume of the liposomes.

     Liposomes are useful for the transfer and delivery of
active ingredients to the site of action. Because the
liposomal membrane is structurally similar to biological

membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active

5 agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations.

10 Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

15 Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications

20 resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds

25 to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, *1987*, *147*, 980-985).

30 Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped

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within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and

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cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to

5 deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. S.T.P.Pharma. Sci., **1994**, 4, 6, 466).

10 Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized

15 lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol

20 (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized

25 liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., FEBS Letters, **1987**, 223, 42; Wu et al., Cancer Research, **1993**, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (Ann. N.Y. Acad.

30 Sci., **1987**, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et

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al. (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, *85*, 6949). U.S.
Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*,
disclose liposomes comprising (1) sphingomyelin and (2) the
ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S.
5   Patent No. 5,543,152 (Webb *et al.*) discloses liposomes
comprising sphingomyelin. Liposomes comprising 1,2-*sn*-
dimyristoylphosphatidylcholine are disclosed in WO 97/13499
(Lim *et al.*).

Many liposomes comprising lipids derivatized with one or
10   more hydrophilic polymers, and methods of preparation
thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem.
Soc. Jpn.*, **1980**, *53*, 2778) described liposomes comprising a
nonionic detergent, $2C_{12}15G$, that contains a PEG moiety.
Illum *et al.* (*FEBS Lett.*, **1984**, *167*, 79) noted that
15   hydrophilic coating of polystyrene particles with polymeric
glycols results in significantly enhanced blood half-lives.
Synthetic phospholipids modified by the attachment of
carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are
described by Sears (U.S. Patent Nos. 4,426,330 and
20   4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, *268*, 235)
described experiments demonstrating that liposomes comprising
phosphatidylethanolamine (PE) derivatized with PEG or PEG
stearate have significant increases in blood circulation
half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*,
25   **1990**, *1029*, 91) extended such observations to other PEG-
derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the
combination of distearoylphosphatidylethanolamine (DSPE) and
PEG. Liposomes having covalently bound PEG moieties on their
external surface are described in European Patent No. EP 0
30   445 131 B1 and WO 90/04384 to Fisher. Liposome compositions
containing 1-20 mole percent of PE derivatized with PEG, and
methods of use thereof, are described by Woodle *et al.* (U.S.
Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S.

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Patent No. 5,213,804 and European Patent No. EP 0 496 813

B1). Liposomes comprising a number of other lipid-polymer

conjugates are disclosed in WO 91/05545 and U.S. Patent No.

5,225,212 (both to Martin *et al*.) and in WO 94/20073

5   (Zalipsky *et al*.) Liposomes comprising PEG-modified ceramide

lipids are described in WO 96/10391 (Choi *et al*.). U.S.

Patent Nos. 5,540,935 (Miyazaki *et al*.) and 5,556,948 (Tagawa

et al.) describe PEG-containing liposomes that can be further

derivatized with functional moieties on their surfaces.

10   A limited number of liposomes comprising nucleic acids

are known in the art. WO 96/40062 to Thierry *et al*.

discloses methods for encapsulating high molecular weight

nucleic acids in liposomes. U.S. Patent No. 5,264,221 to

Tagawa *et al*. discloses protein-bonded liposomes and asserts

15   that the contents of such liposomes may include an antisense

RNA. U.S. Patent No. 5,665,710 to Rahman *et al*. describes

certain methods of encapsulating oligodeoxynucleotides in

liposomes. WO 97/04787 to Love *et al*. discloses liposomes

comprising antisense oligonucleotides targeted to the raf

20   gene.

          Transfersomes are yet another type of liposomes, and are

highly deformable lipid aggregates which are attractive

candidates for drug delivery vehicles. Transfersomes may be

described as lipid droplets which are so highly deformable

25   that they are easily able to penetrate through pores which

are smaller than the droplet. Transfersomes are adaptable to

the environment in which they are used, *e.g.* they are self-

optimizing (adaptive to the shape of pores in the skin),

self-repairing, frequently reach their targets without

30   fragmenting, and often self-loading. To make transfersomes

it is possible to add surface edge-activators, usually

surfactants, to a standard liposomal composition.

Transfersomes have been used to deliver serum albumin to the

skin. The transfersome-mediated delivery of serum albumin
has been shown to be as effective as subcutaneous injection
of a solution containing serum albumin.

Surfactants find wide application in formulations such
as emulsions (including microemulsions) and liposomes. The
most common way of classifying and ranking the properties of
the many different types of surfactants, both natural and
synthetic, is by the use of the hydrophile/lipophile balance
(HLB). The nature of the hydrophilic group (also known as
the "head") provides the most useful means for categorizing
the different surfactants used in formulations (Rieger, in
Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York,
NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is
classified as a nonionic surfactant. Nonionic surfactants
find wide application in pharmaceutical and cosmetic products
and are usable over a wide range of pH values. In general
their HLB values range from 2 to about 18 depending on their
structure. Nonionic surfactants include nonionic esters such
as ethylene glycol esters, propylene glycol esters, glyceryl
esters, polyglyceryl esters, sorbitan esters, sucrose esters,
and ethoxylated esters. Nonionic alkanolamides and ethers
such as fatty alcohol ethoxylates, propoxylated alcohols, and
ethoxylated/propoxylated block polymers are also included in
this class. The polyoxyethylene surfactants are the most
popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge
when it is dissolved or dispersed in water, the surfactant is
classified as anionic. Anionic surfactants include
carboxylates such as soaps, acyl lactylates, acyl amides of
amino acids, esters of sulfuric acid such as alkyl sulfates
and ethoxylated alkyl sulfates, sulfonates such as alkyl
benzene sulfonates, acyl isethionates, acyl taurates and

sulfosuccinates, and phosphates. The most important members
of the anionic surfactant class are the alkyl sulfates and
the soaps.

If the surfactant molecule carries a positive charge
5   when it is dissolved or dispersed in water, the surfactant is
classified as cationic. Cationic surfactants include
quaternary ammonium salts and ethoxylated amines. The
quaternary ammonium salts are the most used members of this
class.

10   If the surfactant molecule has the ability to carry
either a positive or negative charge, the surfactant is
classified as amphoteric. Amphoteric surfactants include
acrylic acid derivatives, substituted alkylamides, N-
alkylbetaines and phosphatides.

15   The use of surfactants in drug products, formulations
and in emulsions has been reviewed (Rieger, in *Pharmaceutical
Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p.
285).

20   Penetration Enhancers

In one embodiment, the present invention employs various
penetration enhancers to effect the efficient delivery of
nucleic acids, particularly oligonucleotides, to the skin of
animals. Most drugs are present in solution in both ionized
25   and nonionized forms. However, usually only lipid soluble or
lipophilic drugs readily cross cell membranes. It has been
discovered that even non-lipophilic drugs may cross cell
membranes if the membrane to be crossed is treated with a
penetration enhancer. In addition to aiding the diffusion of
30   non-lipophilic drugs across cell membranes, penetration
enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to
one of five broad categories, *i.e.*, surfactants, fatty acids,

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bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration enhancers are described below in

5   greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce

10   the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example,

15   sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

20   Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-

25   monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (e.g., methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (i.e., oleate,

30   laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in*

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Therapeutic Drug Carrier Systems, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., **1992**, *263*, 25; Yamashita *et al.*, J. Pharm. Sci., **1990**, *79*, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as

compounds that remove metallic ions from solution by forming
complexes therewith, with the result that absorption of
oligonucleotides through the mucosa is enhanced. With
regards to their use as penetration enhancers in the present
5   invention, chelating agents have the added advantage of also
serving as DNase inhibitors, as most characterized DNA
nucleases require a divalent metal ion for catalysis and are
thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*,
1993, *618*, 315-339). Chelating agents of the invention
10   include but are not limited to disodium
ethylenediaminetetraacetate (EDTA), citric acid, salicylates
(*e.g.*, sodium salicylate, 5-methoxysalicylate and
homovanilate), *N*-acyl derivatives of collagen, laureth-9 and
N-amino acyl derivatives of beta-diketones (enamines)(Lee *et*
15   *al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*,
1991, page 92; Muranishi, *Critical Reviews in Therapeutic*
Drug Carrier Systems, **1990**, *7*, 1-33; Buur *et al.*, *J. Control*
Rel., **1990**, *14*, 43-51).

20   Non-chelating non-surfactants: As used herein, non-
chelating non-surfactant penetration enhancing compounds can
be defined as compounds that demonstrate insignificant
activity as chelating agents or as surfactants but that
nonetheless enhance absorption of oligonucleotides through
25   the alimentary mucosa (Muranishi, *Critical Reviews in*
Therapeutic Drug Carrier Systems, **1990**, *7*, 1-33). This class
of penetration enhancers include, for example, unsaturated
cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone
derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug*
30   *Carrier Systems*, **1991**, page 92); and non-steroidal anti-
inflammatory agents such as diclofenac sodium, indomethacin
and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*,
1987, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S.

5   Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration

10 of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

15   Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as

20 a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound,

25 typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For

30 example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-

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disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5,* 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6,* 177-183).

5   Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an
10   animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.,* when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical
15   carriers include, but are not limited to, binding agents (*e.g.,* pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.,* lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or
20   calcium hydrogen phosphate, *etc.*); lubricants (*e.g.,* magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.,* starch, sodium
25   starch glycolate, *etc.*); and wetting agents (*e.g.,* sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used
30   to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium

stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-
5   aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral
10   administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium
15   stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components
20   The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible,
25   pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes,
30   flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the

present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings,

5   flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for

10 example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other

15 chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine

20 arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil,

25 methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine,

30 taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp.

1206-1228, Berkow *et al.*, eds., Rahway, N.J.   When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow *et al.*, eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body

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of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can
5 generally be estimated based on $EC_{50}s$ found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art
10 can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state,
15 wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred
20 embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

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EXAMPLES

Example 1

5   **Nucleoside Phosphoramidites for Oligonucleotide Synthesis**
Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl
phosphoramidites were purchased from commercial sources (e.g.
Chemgenes, Needham MA or Glen Research, Inc. Sterling VA).

10 Other 2'-O-alkoxy substituted nucleoside amidites are
prepared as described in U.S. Patent 5,506,351, herein
incorporated by reference. For oligonucleotides synthesized
using 2'-alkoxy amidites, optimized synthesis cycles were
developed that incorporate multiple steps coupling longer

15 wait times relative to standard synthesis cycles.

The following abbreviations are used in the text: thin
layer chromatography (TLC), melting point (MP), high pressure
liquid chromatography (HPLC), Nuclear Magnetic Resonance
(NMR), argon (Ar), methanol (MeOH), dichloromethane (CH_2Cl_2),

20 triethylamine (TEA), dimethyl formamide (DMF), ethyl acetate
(EtOAc), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF).

Oligonucleotides containing 5-methyl-2'-deoxycytidine
(5-Me-dC) nucleotides were synthesized according to published
methods (Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*,

25 3197-3203) using commercially available phosphoramidites
(Glen Research, Sterling VA or ChemGenes, Needham MA) or
prepared as follows:

Preparation of 5'-O-Dimethoxytrityl-thymidine intermediate
30 **for 5-methyl dC amidite**

To a 50 L glass reactor equipped with air stirrer and Ar
gas line was added thymidine (1.00 kg, 4.13 mol) in anhydrous
pyridine (6 L) at ambient temperature. Dimethoxytrityl (DMT)

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chloride (1.47 kg, 4.34 mol, 1.05 eq) was added as a solid in four portions over 1 h. After 30 min, TLC indicated approx. 95% product, 2% thymidine, 5% DMT reagent and by-products and 2 % 3',5'-bis DMT product (R_f in EtOAc 0.45, 0.05, 0.98, 0.95

5   respectively). Saturated sodium bicarbonate (4 L) and CH_2Cl_2 were added with stirring (pH of the aqueous layer 7.5). An additional 18 L of water was added, the mixture was stirred, the phases were separated, and the organic layer was transferred to a second 50 L vessel. The aqueous layer was

10 extracted with additional CH_2Cl_2 (2 x 2 L). The combined organic layer was washed with water (10 L) and then concentrated in a rotary evaporator to approx. 3.6 kg total weight. This was redissolved in CH_2Cl_2 (3.5 L), added to the reactor followed by water (6 L) and hexanes (13 L). The

15 mixture was vigorously stirred and seeded to give a fine white suspended solid starting at the interface. After stirring for 1 h, the suspension was removed by suction through a 1/2" diameter teflon tube into a 20 L suction flask, poured onto a 25 cm Coors Buchner funnel, washed with

20 water (2 x 3 L) and a mixture of hexanes- CH_2Cl_2 (4:1, 2x3 L) and allowed to air dry overnight in pans (1" deep). This was further dried in a vacuum oven (75°C, 0.1 mm Hg, 48 h) to a constant weight of 2072 g (93%) of a white solid, (mp 122-124°C). TLC indicated a trace contamination of the bis DMT

25 product. NMR spectroscopy also indicated that 1-2 mole percent pyridine and about 5 mole percent of hexanes was still present.

Preparation of 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine

30 **intermediate for 5-methyl-dC amidite**

      To a 50 L Schott glass-lined steel reactor equipped with an electric stirrer, reagent addition pump (connected to an addition funnel), heating/cooling system, internal

thermometer and an Ar gas line was added 5'-O-dimethoxytrityl-thymidine (3.00 kg, 5.51 mol), anhydrous acetonitrile (25 L) and TEA (12.3 L, 88.4 mol, 16 eq). The mixture was chilled with stirring to -10°C internal

5   temperature (external -20°C). Trimethylsilylchloride (2.1 L, 16.5 mol, 3.0 eq) was added over 30 minutes while maintaining the internal temperature below -5°C, followed by a wash of anhydrous acetonitrile (1 L). Note: the reaction is mildly exothermic and copious hydrochloric acid fumes form over the

10   course of the addition. The reaction was allowed to warm to 0°C and the reaction progress was confirmed by TLC (EtOAc-hexanes 4:1; R_f 0.43 to 0.84 of starting material and silyl product, respectively). Upon completion, triazole (3.05 kg, 44 mol, 8.0 eq) was added the reaction was cooled to -20°C

15   internal temperature (external -30°C). Phosphorous oxychloride (1035 mL, 11.1 mol, 2.01 eq) was added over 60 min so as to maintain the temperature between -20°C and -10°C during the strongly exothermic process, followed by a wash of anhydrous acetonitrile (1 L). The reaction was warmed to 0

20   °C and stirred for 1 h. TLC indicated a complete conversion to the triazole product (R_f 0.83 to 0.34 with the product spot glowing in long wavelength UV light). The reaction mixture was a peach-colored thick suspension, which turned darker red upon warming without apparent decomposition. The reaction

25   was cooled to -15°C internal temperature and water (5 L) was slowly added at a rate to maintain the temperature below +10°C in order to quench the reaction and to form a homogenous solution. (Caution: this reaction is initially very strongly exothermic). Approximately one-half of the

30   reaction volume (22 L) was transferred by air pump to another vessel, diluted with EtOAc (12 L) and extracted with water (2 x 8 L). The combined water layers were back-extracted with EtOAc (6 L). The water layer was discarded and the organic

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layers were concentrated in a 20 L rotary evaporator to an oily foam. The foam was coevaporated with anhydrous acetonitrile (4 L) to remove EtOAc. (note: dioxane may be used instead of anhydrous acetonitrile if dried to a hard

5   foam). The second half of the reaction was treated in the same way. Each residue was dissolved in dioxane (3 L) and concentrated ammonium hydroxide (750 mL) was added. A homogenous solution formed in a few minutes and the reaction was allowed to stand overnight (although the reaction is

10   complete within 1 h).

TLC indicated a complete reaction (product R_f 0.35 in EtOAc-MeOH 4:1). The reaction solution was concentrated on a rotary evaporator to a dense foam. Each foam was slowly redissolved in warm EtOAc (4 L; 50°C), combined in a 50 L

15   glass reactor vessel, and extracted with water (2 x 4L) to remove the triazole by-product. The water was back-extracted with EtOAc (2 L). The organic layers were combined and concentrated to about 8 kg total weight, cooled to 0°C and seeded with crystalline product. After 24 hours, the first

20   crop was collected on a 25 cm Coors Buchner funnel and washed repeatedly with EtOAc (3 x 3L) until a white powder was left and then washed with ethyl ether (2 x 3L). The solid was put in pans (1" deep) and allowed to air dry overnight. The filtrate was concentrated to an oil, then redissolved in

25   EtOAc (2 L), cooled and seeded as before. The second crop was collected and washed as before (with proportional solvents) and the filtrate was first extracted with water (2 x 1L) and then concentrated to an oil. The residue was dissolved in EtOAc (1 L) and yielded a third crop which was

30   treated as above except that more washing was required to remove a yellow oily layer.

After air-drying, the three crops were dried in a vacuum oven (50°C, 0.1 mm Hg, 24 h) to a constant weight (1750, 600

and 200 g, respectively) and combined to afford 2550 g (85%)
of a white crystalline product (MP 215-217°C) when TLC and
NMR spectroscopy indicated purity. The mother liquor still
contained mostly product (as determined by TLC) and a small
5 amount of triazole (as determined by NMR spectroscopy), bis
DMT product and unidentified minor impurities. If desired,
the mother liquor can be purified by silica gel
chromatography using a gradient of MeOH (0-25%) in EtOAc to
further increase the yield.
10

**Preparation of 5'-O-Dimethoxytrityl-2'-deoxy-N4-benzoyl-5-
methylcytidine penultimate intermediate for 5-methyl dC
amidite**

Crystalline 5'-O-dimethoxytrityl-5-methyl-2'-
15 deoxycytidine (2000 g, 3.68 mol) was dissolved in anhydrous
DMF (6.0 kg) at ambient temperature in a 50 L glass reactor
vessel equipped with an air stirrer and argon line. Benzoic
anhydride (Chem Impex not Aldrich, 874 g, 3.86 mol, 1.05 eq)
was added and the reaction was stirred at ambient temperature
20 for 8 h. TLC (CH_2Cl_2-EtOAc; CH_2Cl_2-EtOAc 4:1; R_f 0.25)
indicated approx. 92% complete reaction. An additional
amount of benzoic anhydride (44 g, 0.19 mol) was added.
After a total of 18 h, TLC indicated approx. 96% reaction
completion. The solution was diluted with EtOAc (20 L), TEA
25 (1020 mL, 7.36 mol, ca 2.0 eq) was added with stirring, and
the mixture was extracted with water (15 L, then 2 x 10 L).
The aqueous layer was removed (no back-extraction was needed)
and the organic layer was concentrated in 2 x 20 L rotary
evaporator flasks until a foam began to form. The residues
30 were coevaporated with acetonitrile (1.5 L each) and dried
(0.1 mm Hg, 25°C, 24 h) to 2520 g of a dense foam. High
pressure liquid chromatography (HPLC) revealed a

contamination of 6.3% of N4, 3'-O-dibenzoyl product, but very
little other impurities.

THe product was purified by Biotage column
chromatography (5 kg Biotage) prepared with 65:35:1 hexanes-

5   EtOAc-TEA (4L). The crude product (800 g),dissolved in CH_2Cl_2
(2 L), was applied to the column. The column was washed with
the 65:35:1 solvent mixture (20 kg), then 20:80:1 solvent
mixture (10 kg), then 99:1 EtOAc:TEA (17kg). The fractions
containing the product were collected, and any fractions

10   containing the product and impurities were retained to be
resubjected to column chromatography. The column was re-
equilibrated with the original 65:35:1 solvent mixture (17
kg). A second batch of crude product (840 g) was applied to
the column as before. The column was washed with the

15   following solvent gradients: 65:35:1 (9 kg), 55:45:1 (20
kg), 20:80:1 (10 kg), and 99:1 EtOAc:TEA(15 kg). The column
was reequilibrated as above, and a third batch of the crude
product (850 g) plus impure fractions recycled from the two
previous columns (28 g) was purified following the procedure

20   for the second batch. The fractions containing pure product
combined and concentrated on a 20L rotary evaporator, co-
evaporated with acetontirile (3 L) and dried (0.1 mm Hg, 48
h, 25°C) to a constant weight of 2023 g (85%) of white foam
and 20 g of slightly contaminated product from the third run.

25   HPLC indicated a purity of 99.8% with the balance as the
diBenzoyl product.

[5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N^4-
benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-

30   diisopropylphosphoramidite (5-methyl dC amidite)
5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N^4-benzoyl-
5-methylcytidine (998 g, 1.5 mol) was dissolved in anhydrous
DMF (2 L). The solution was co-evaporated with toluene (300

ml) at 50°C under reduced pressure, then cooled to room
temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite
(680 g, 2.26 mol) and tetrazole (52.5 g, 0.75 mol) were
added. The mixture was shaken until all tetrazole was
5   dissolved, N-methylimidazole (15 ml) was added and the
mixture was left at room temperature for 5 hours. TEA (300
ml) was added, the mixture was diluted with DMF (2.5 L) and
water (600 ml), and extracted with hexane (3 x 3 L). The
mixture was diluted with water (1.2 L) and extracted with a
10   mixture of toluene (7.5 L) and hexane (6 L). The two layers
were separated, the upper layer was washed with DMF-water
(7:3 v/v, 3 x 2 L) and water (3 x 2 L), and the phases were
separated. The organic layer was dried (Na$_2$SO$_4$), filtered and
rotary evaporated. The residue was co-evaporated with
15   acetonitrile (2 x 2 L) under reduced pressure and dried to a
constant weight (25 °C, 0.1mm Hg, 40 h) to afford 1250 g an
off-white foam solid (96%).

2'-Fluoro amidites

20   **2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described
previously [Kawasaki, *et. al.*, *J. Med. Chem.*, **1993**, *36*, 831-
841] and United States patent 5,670,633, herein incorporated
by reference. The preparation of 2'-fluoropyrimidines
25   containing a 5-methyl substitution are described in US Patent
5,861,493. Briefly, the protected nucleoside N6-benzoyl-2'-
deoxy-2'-fluoroadenosine was synthesized utilizing
commercially available 9-beta-D-arabinofuranosyladenine as
starting material and whereby the 2'-alpha-fluoro atom is
30   introduced by a S$_N$2-displacement of a 2'-beta-triflate group.
Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was
selectively protected in moderate yield as the 3',5'-
ditetrahydropyranyl (THP) intermediate. Deprotection of the

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THP and N6-benzoyl groups was accomplished using standard methodologies to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate isobutyryl-arabinofuranosylguanosine. Alternatively, isobutyryl-arabinofuranosylguanosine was prepared as described by Ross et al., (Nucleosides & Nucleosides, 16, 1645, **1997**). Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give isobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites (otherwise known as MOE amidites) are prepared as follows, or alternatively, as per the methods of Martin, P., (Helvetica Chimica Acta, **1995**, *78*, 486-504).

Preparation of 2'-O-(2-methoxyethyl)-5-methyluridine intermediate

2,2'-Anhydro-5-methyl-uridine (2000 g, 8.32 mol), tris(2-methoxyethyl)borate (2504 g, 10.60 mol), sodium bicarbonate (60 g, 0.70 mol) and anhydrous 2-methoxyethanol (5 L) were combined in a 12 L three necked flask and heated to 130 °C (internal temp) at atmospheric pressure, under an argon atmosphere with stirring for 21 h. TLC indicated a complete reaction. The solvent was removed under reduced pressure until a sticky gum formed (50-85°C bath temp and 100-11 mm Hg) and the residue was redissolved in water (3 L) and heated to boiling for 30 min in order the hydrolyze the borate esters. The water was removed under reduced pressure until a foam began to form and then the process was repeated. HPLC indicated about 77% product, 15% dimer (5' of product attached to 2' of starting material) and unknown derivatives, and the balance was a single unresolved early eluting peak.

The gum was redissolved in brine (3 L), and the flask was rinsed with additional brine (3 L). The combined aqueous solutions were extracted with chloroform (20 L) in a heavier-than continuous extractor for 70 h. The chloroform layer was concentrated by rotary evaporation in a 20 L flask to a sticky foam (2400 g). This was coevaporated with MeOH (400 mL) and EtOAc (8 L) at 75°C and 0.65 atm until the foam dissolved at which point the vacuum was lowered to about 0.5 atm. After 2.5 L of distillate was collected a precipitate

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began to form and the flask was removed from the rotary
evaporator and stirred until the suspension reached ambient
temperature. EtOAc (2 L) was added and the slurry was
filtered on a 25 cm table top Buchner funnel and the product

5   was washed with EtOAc (3 x 2 L). The bright white solid was
air dried in pans for 24 h then further dried in a vacuum
oven (50°C, 0.1 mm Hg, 24 h) to afford 1649 g of a white
crystalline solid (mp 115.5-116.5°C).

The brine layer in the 20 L continuous extractor was

10   further extracted for 72 h with recycled chloroform. The
chloroform was concentrated to 120 g of oil and this was
combined with the mother liquor from the above filtration
(225 g), dissolved in brine (250 mL) and extracted once with
chloroform (250 mL). The brine solution was continuously

15   extracted and the product was crystallized as described above
to afford an additional 178 g of crystalline product
containing about 2% of thymine. The combined yield was 1827 g
(69.4%). HPLC indicated about 99.5% purity with the balance
being the dimer.

20

**Preparation of 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine
penultimate intermediate**

In a 50 L glass-lined steel reactor, 2'-O-(2-
methoxyethyl)-5-methyl-uridine (MOE-T, 1500 g, 4.738 mol),

25   lutidine (1015 g, 9.476 mol) were dissolved in anhydrous
acetonitrile (15 L). The solution was stirred rapidly and
chilled to -10°C (internal temperature).
Dimethoxytriphenylmethyl chloride (1765.7 g, 5.21 mol) was
added as a solid in one portion. The reaction was allowed to

30   warm to -2°C over 1 h. (Note: The reaction was monitored
closely by TLC (EtOAc) to determine when to stop the reaction
so as to not generate the undesired bis-DMT substituted side
product). The reaction was allowed to warm from -2 to 3°C

over 25 min. then quenched by adding MeOH (300 mL) followed
after 10 min by toluene (16 L) and water (16 L). The
solution was transferred to a clear 50 L vessel with a bottom
outlet, vigorously stirred for 1 minute, and the layers

5 separated. The aqueous layer was removed and the organic
layer was washed successively with 10% aqueous citric acid (8
L) and water (12 L). The product was then extracted into the
aqueous phase by washing the toluene solution with aqueous
sodium hydroxide (0.5N, 16 L and 8 L). The combined aqueous

10 layer was overlayed with toluene (12 L) and solid citric acid
(8 moles, 1270 g) was added with vigorous stirring to lower
the pH of the aqueous layer to 5.5 and extract the product
into the toluene. The organic layer was washed with water
(10 L) and TLC of the organic layer indicated a trace of DMT-

15 O-Me, bis DMT and dimer DMT.

     The toluene solution was applied to a silica gel column
(6 L sintered glass funnel containing approx. 2 kg of silica
gel slurried with toluene (2 L) and TEA(25 mL)) and the
fractions were eluted with toluene (12 L) and EtOAc (3 x 4 L)

20 using vacuum applied to a filter flask placed below the
column. The first EtOAc fraction containing both the desired
product and impurities were resubjected to column
chromatography as above. The clean fractions were combined,
rotary evaporated to a foam, coevaporated with acetonitrile

25 (6 L) and dried in a vacuum oven (0.1 mm Hg, 40 h, 40°C) to
afford 2850 g of a white crisp foam. NMR spectroscopy
indicated a 0.25 mole % remainder of acetonitrile (calculates
to be approx. 47 g) to give a true dry weight of 2803 g
(96%). HPLC indicated that the product was 99.41% pure, with

30 the remainder being 0.06 DMT-O-Me, 0.10 unknown, 0.44 bis
DMT, and no detectable dimer DMT or 3'-O-DMT.

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Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-5-methyluridin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE T amidite)

5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-5-methyluridine (1237 g, 2.0 mol) was dissolved in anhydrous DMF (2.5 L). The solution was co-evaporated with toluene (200 ml) at 50°C under reduced pressure, then cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (900 g, 3.0 mol) and tetrazole (70 g, 1.0 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (20 ml) was added and the solution was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (3.5 L) and water (600 ml) and extracted with hexane (3 x 3L). The mixture was diluted with water (1.6 L) and extracted with the mixture of toluene (12 L) and hexanes (9 L). The upper layer was washed with DMF-water (7:3 v/v, 3x3 L) and water (3x3 L). The organic layer was dried (Na₂SO₄), filtered and evaporated. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried in a vacuum oven (25°C, 0.1mm Hg, 40 h) to afford 1526 g of an off-white foamy solid (95%).

Preparation of 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate

To a 50 L Schott glass-lined steel reactor equipped with an electric stirrer, reagent addition pump (connected to an addition funnel), heating/cooling system, internal thermometer and argon gas line was added 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methyl-uridine (2.616 kg, 4.23 mol, purified by base extraction only and no scrub column), anhydrous acetonitrile (20 L), and TEA (9.5 L, 67.7 mol, 16 eq). The mixture was chilled with stirring to -10°C

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internal temperature (external -20°C).
Trimethylsilylchloride (1.60 L, 12.7 mol, 3.0 eq) was added
over 30 min. while maintaining the internal temperature below
-5°C, followed by a wash of anhydrous acetonitrile (1 L).

5 (Note: the reaction is mildly exothermic and copious
hydrochloric acid fumes form over the course of the
addition). The reaction was allowed to warm to 0°C and the
reaction progress was confirmed by TLC (EtOAc, R_f 0.68 and
0.87 for starting material and silyl product, respectively).

10 Upon completion, triazole (2.34 kg, 33.8 mol, 8.0 eq) was
added the reaction was cooled to -20°C internal temperature
(external -30°C). Phosphorous oxychloride (793 mL, 8.51 mol,
2.01 eq) was added slowly over 60 min so as to maintain the
temperature between -20°C and -10°C (note: strongly

15 exothermic), followed by a wash of anhydrous acetonitrile (1
L). The reaction was warmed to 0°C and stirred for 1 h, at
which point it was an off-white thick suspension. TLC
indicated a complete conversion to the triazole product
(EtOAc, R_f 0.87 to 0.75 with the product spot glowing in long

20 wavelength UV light). The reaction was cooled to -15°C and
water (5 L) was slowly added at a rate to maintain the
temperature below +10°C in order to quench the reaction and
to form a homogenous solution. (Caution: this reaction is
initially very strongly exothermic). Approximately one-half

25 of the reaction volume (22 L) was transferred by air pump to
another vessel, diluted with EtOAc (12 L) and extracted with
water (2 x 8 L). The second half of the reaction was treated
in the same way. The combined aqueous layers were back-
extracted with EtOAc (8 L) The organic layers were combined

30 and concentrated in a 20 L rotary evaporator to an oily foam.
The foam was coevaporated with anhydrous acetonitrile (4 L)
to remove EtOAc. (note: dioxane may be used instead of
anhydrous acetonitrile if dried to a hard foam). The residue

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was dissolved in dioxane (2 L) and concentrated ammonium hydroxide (750 mL) was added. A homogenous solution formed in a few minutes and the reaction was allowed to stand overnight

5   TLC indicated a complete reaction (CH$_2$Cl$_2$-acetone-MeOH, 20:5:3, R$_f$ 0.51). The reaction solution was concentrated on a rotary evaporator to a dense foam and slowly redissolved in warm CH$_2$Cl$_2$ (4 L, 40°C) and transferred to a 20 L glass extraction vessel equipped with a air-powered stirrer. The

10   organic layer was extracted with water (2 x 6 L) to remove the triazole by-product. (Note: In the first extraction an emulsion formed which took about 2 h to resolve). The water layer was back-extracted with CH$_2$Cl$_2$ (2 x 2 L), which in turn was washed with water (3 L). The combined organic layer was

15   concentrated in 2 x 20 L flasks to a gum and then recrystallized from EtOAc seeded with crystalline product. After sitting overnight, the first crop was collected on a 25 cm Coors Buchner funnel and washed repeatedly with EtOAc until a white free-flowing powder was left (about 3 x 3 L).

20   The filtrate was concentrated to an oil recrystallized from EtOAc, and collected as above. The solid was air-dried in pans for 48 h, then further dried in a vacuum oven (50°C, 0.1mm Hg, 17 h) to afford 2248 g of a bright white, dense solid (86%). An HPLC analysis indicated both crops to be

25   99.4% pure and NMR spectroscopy indicated only a faint trace of EtOAc remained.

Preparation of 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-N4-benzoyl-5-methyl-cytidine penultimate intermediate:

30   Crystalline 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methyl-cytidine (1000 g, 1.62 mol) was suspended in anhydrous DMF (3 kg) at ambient temperature and stirred under an Ar atmosphere. Benzoic anhydride (439.3 g, 1.94 mol) was

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added in one portion. The solution clarified after 5 hours and was stirred for 16 h. HPLC indicated 0.45% starting material remained (as well as 0.32% N4, 3'-O-bis Benzoyl).

An additional amount of benzoic anhydride (6.0 g, 0.0265 mol)
5   was added and after 17 h, HPLC indicated no starting material was present. TEA (450 mL, 3.24 mol) and toluene (6 L) were added with stirring for 1 minute. The solution was washed with water (4 x 4 L), and brine (2 x 4 L). The organic layer was partially evaporated on a 20 L rotary evaporator to
10 remove 4 L of toluene and traces of water. HPLC indicated that the bis benzoyl side product was present as a 6% impurity. The residue was diluted with toluene (7 L) and anhydrous DMSO (200 mL, 2.82 mol) and sodium hydride (60% in oil, 70 g, 1.75 mol) was added in one portion with stirring
15 at ambient temperature over 1 h. The reaction was quenched by slowly adding then washing with aqueous citric acid (10%, 100 mL over 10 min, then 2 x 4 L), followed by aqueous sodium bicarbonate (2%, 2 L), water (2 x 4 L) and brine (4 L). The organic layer was concentrated on a 20 L rotary evaporator to
20 about 2 L total volume. The residue was purified by silica gel column chromatography (6 L Buchner funnel containing 1.5 kg of silica gel wetted with a solution of EtOAc-hexanes-TEA(70:29:1)). The product was eluted with the same solvent (30 L) followed by straight EtOAc (6 L). The fractions
25 containing the product were combined, concentrated on a rotary evaporator to a foam and then dried in a vacuum oven (50°C, 0.2 mm Hg, 8 h) to afford 1155 g of a crisp, white foam (98%). HPLC indicated a purity of >99.7%.

30   **Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N^4-benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE 5-Me-C amidite)**

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5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methylcytidine (1082 g, 1.5 mol) was dissolved in anhydrous DMF (2 L) and co-evaporated with toluene (300 ml) at 50 °C under reduced pressure. The mixture

5   was cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (680 g, 2.26 mol) and tetrazole (52.5 g, 0.75 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (30 ml) was added, and the mixture was left at room

10   temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (1 L) and water (400 ml) and extracted with hexane (3 x 3 L). The mixture was diluted with water (1.2 L) and extracted with a mixture of toluene (9 L) and hexanes (6 L). The two layers were separated and the upper

15   layer was washed with DMF-water (60:40 v/v, 3 x 3 L) and water (3 x 2 L). The organic layer was dried (Na$_2$SO$_4$), filtered and evaporated. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg, 40 h) to afford 1336 g of an

20   off-white foam (97%).

Preparation of [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^6-benzoyladenosin-3'-*O*-yl]-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (MOE A amdite)

5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-

25   methoxyethyl)-N^6-benzoyladenosine (purchased from Reliable Biopharmaceutical, St. Lois, MO), 1098 g, 1.5 mol) was dissolved in anhydrous DMF (3 L) and co-evaporated with toluene (300 ml) at 50 °C. The mixture was cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite

30   (680 g, 2.26 mol) and tetrazole (78.8 g, 1.24 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (30 ml) was added, and mixture was left at room temperature for 5 hours. TEA (300 ml) was

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added, the mixture was diluted with DMF (1 L) and water (400 ml) and extracted with hexanes (3 x 3 L). The mixture was diluted with water (1.4 L) and extracted with the mixture of toluene (9 L) and hexanes (6 L). The two layers were

5   separated and the upper layer was washed with DMF-water (60:40, v/v, 3 x 3 L) and water (3 x 2 L). The organic layer was dried (Na_2SO_4), filtered and evaporated to a sticky foam. The residue was co-evaporated with acetonitrile (2.5 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg,

10   40 h) to afford 1350 g of an off-white foam solid (96%).

Prepartion of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N^4-isobutyrylguanosin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE G amidite)

15   5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N^4-isobutyrlguanosine (purchased from Reliable Biopharmaceutical, St. Louis, MO, 1426 g, 2.0 mol) was dissolved in anhydrous DMF (2 L). The solution was co-evaporated with toluene (200 ml) at 50 °C, cooled to room

20   temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (900 g, 3.0 mol) and tetrazole (68 g, 0.97 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (30 ml) was added, and the mixture was left at room temperature for 5 hours. TEA (300 ml) was added, the

25   mixture was diluted with DMF (2 L) and water (600 ml) and extracted with hexanes (3 x 3 L). The mixture was diluted with water (2 L) and extracted with a mixture of toluene (10 L) and hexanes (5 L). The two layers were separated and the upper layer was washed with DMF-water (60:40, v/v, 3x3 L).

30   EtOAc (4 L) was added and the solution was washed with water (3 x 4 L). The organic layer was dried (Na_2SO_4), filtered and evaporated to approx. 4 kg. Hexane (4 L) was added, the mixture was shaken for 10 min, and the supernatant liquid was

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decanted. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg, 40 h) to afford 1660 g of an off-white foamy solid (91%).

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites (also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (R_f 0.22, EtOAc) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between CH_2Cl_2 (1 L) and saturated sodium bicarbonate (2 x 1 L) and brine (1 L). The organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of EtOAc and ethyl ether (600mL) and cooling the

solution to -10°C afforded a white crystalline solid which was collected by filtration, washed with ethyl ether (3 x2 00 mL) and dried (40°C, 1mm Hg, 24 h) to afford 149g of white solid (74.8%). TLC and NMR spectroscopy were consistent with pure
5   product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In the fume hood, ethylene glycol (350 mL, excess) was
10   added cautiously with manual stirring to a 2 L stainless steel pressure reactor containing borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). (Caution : evolves hydrogen gas). 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq)
15   were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient temperature and opened. TLC (EtOAc, R_f 0.67 for desired product and R_f
20   0.82 for ara-T side product) indicated about 70% conversion to the product. The solution was concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. (Alternatively, once the THF has evaporated the
25   solution can be diluted with water and the product extracted into EtOAc). The residue was purified by column chromatography (2kg silica gel, EtOAc-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, evaporated and dried to afford 84 g of a white crisp foam (50%),
30   contaminated starting material (17.4g, 12% recovery) and pure reusable starting material (20g, 13% recovery). TLC and NMR spectroscopy were consistent with 99% pure product.

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2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with
5   triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol) and dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dissolved in dry THF (369.8mL, Aldrich, sure seal bottle). Diethyl-azodicarboxylate
10   (6.98mL, 44.36mmol) was added dropwise to the reaction mixture with the rate of addition maintained such that the resulting deep red coloration is just discharged before adding the next drop. The reaction mixture was stirred for 4 hrs., after which time TLC (EtOAc:hexane, 60:40) indicated
15   that the reaction was complete. The solvent was evaporated in vacuuo and the residue purified by flash column chromatography (eluted with 60:40 EtOAc:hexane), to yield 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%) upon rotary
20   evaporation.

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-
25   methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate washed with ice cold CH_2Cl_2, and the combined organic phase was washed with water and brine and dried
30   (anhydrous Na_2SO_4). The solution was filtered and evaporated to afford 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). Formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture

was stirred for 1 h. The solvent was removed under vacuum
and the residue was purified by column chromatography to
yield 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)
ethyl]-5-methyluridine as white foam (1.95 g, 78%) upon
5   rotary evaporation.

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-
10   formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was
dissolved in a solution of 1M pyridinium p-toluenesulfonate
(PPTS) in dry MeOH (30.6mL) and cooled to 10°C under inert
atmosphere. Sodium cyanoborohydride (0.39g, 6.13mmol) was
added and the reaction mixture was stirred. After 10 minutes
15   the reaction was warmed to room temperature and stirred for 2
h. while the progress of the reaction was monitored by TLC
(5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was
added and the product was extracted with EtOAc (2 x 20 mL).
The organic phase was dried over anhydrous Na_2SO_4, filtered,
20   and evaporated to dryness. This entire procedure was
repeated with the resulting residue, with the exception that
formaldehyde (20% w/w, 30 mL, 3.37 mol) was added upon
dissolution of the residue in the PPTS/MeOH solution. After
the extraction and evaporation, the residue was purified by
25   flash column chromatography and (eluted with 5% MeOH in
CH_2Cl_2) to afford 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-
dimethylaminooxyethyl]-5-methyluridine as a white foam
(14.6g, 80%) upon rotary evaporation.

30   ### 2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was
dissolved in dry THF and TEA (1.67mL, 12mmol, dry, stored
over KOH) and added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-

[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol).
The reaction was stirred at room temperature for 24 hrs and
monitored by TLC (5% MeOH in CH_2Cl_2). The solvent was removed
under vacuum and the residue purified by flash column
chromatography (eluted with 10% MeOH in CH_2Cl_2) to afford 2'-
O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%) upon
rotary evaporation of the solvent.

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750 mg,
2.17 mmol) was dried over P_2O_5 under high vacuum overnight at
40°C, co-evaporated with anhydrous pyridine (20 mL), and
dissolved in pyridine (11 mL) under argon atmosphere. 4-
dimethylaminopyridine (26.5 mg, 2.60 mmol) and 4,4'-
dimethoxytrityl chloride (880 mg, 2.60 mmol) were added to
the pyridine solution and the reaction mixture was stirred at
room temperature until all of the starting material had
reacted. Pyridine was removed under vacuum and the residue
was purified by column chromatography (eluted with 10% MeOH
in CH_2Cl_2 containing a few drops of pyridine) to yield 5'-O-
DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g,
80%) upon rotary evaporation.

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-
methyluridine-3'-[(2-cyanoethyl)-N,N-
diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine
(1.08 g, 1.67 mmol) was co-evaporated with toluene (20 mL),
N,N-diisopropylamine tetrazonide (0.29 g, 1.67 mmol) was
added and the mixture was dried over P_2O_5 under high vacuum
overnight at 40°C. This was dissolved in anhydrous
acetonitrile (8.4 mL) and 2-cyanoethyl-N,N,N^1,N^1-
tetraisopropylphosphoramidite (2.12 mL, 6.08 mmol) was added.

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The reaction mixture was stirred at ambient temperature for 4 h under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:EtOAc 1:1). The solvent was evaporated, then the residue was dissolved in EtOAc (70mL)

5   and washed with 5% aqueous NaHCO$_3$ (40mL). The EtOAc layer was dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. The residue obtained was purified by column chromatography (EtOAc as eluent) to afford 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-

10 N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%) upon rotary evaporation.

2'-(Aminooxyethoxy) nucleoside amidites

    2'-(Aminooxyethoxy) nucleoside amidites (also known in

15 the art as 2'-O-(aminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

20 ### N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

    The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside.

25 Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-

30 ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and

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2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-
O-(4,4'-dimethoxytrityl)guanosine which may be reduced to
provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-
hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before

5   the hydroxyl group may be displaced by N-hydroxyphthalimide
via a Mitsunobu reaction, and the protected nucleoside may be
phosphitylated as usual to yield 2-N-isobutyryl-6-O-
diphenylcarbamoyl-2'-O-([2-phthalmidoxy]ethyl)-5'-O-(4,4'-
dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-

10 diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also
known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-

15 O-CH$_2$-O-CH$_2$-N(CH$_2$)$_2$, or 2'-DMAEOE nucleoside amidites) are
prepared as follows. Other nucleoside amidites are prepared
similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

20 2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50
mmol) was slowly added to a solution of borane in tetra-
hydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL
bomb. (Caution: Hydrogen gas evolves as the solid
dissolves). O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol),

25 and sodium bicarbonate (2.5 mg) were added and the bomb was
sealed, placed in an oil bath and heated to 155°C for 26 h.
then cooled to room temperature. The crude solution was
concentrated, the residue was diluted with water (200 mL) and
extracted with hexanes (200 mL). The product was extracted

30 from the aqueous layer with EtOAc (3 x 200 mL) and the
combined organic layers were washed once with water, dried
over anhydrous sodium sulfate, filtered and concentrated.
The residue was purified by silica gel column chromatography

(eluted with 5:100:2 MeOH/CH2Cl2/TEA) as the eluent. The
appropriate fractions were combined and evaporated to afford
the product as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)
      ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylamino-
ethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL),
was added TEA (0.36 mL) and dimethoxytrityl chloride (DMT-Cl,
0.87 g, 2 eq.) and the reaction was stirred for 1 h. The
reaction mixture was poured into water (200 mL) and extracted
with CH2Cl2 (2 x 200 mL). The combined CH2Cl2 layers were
washed with saturated NaHCO3 solution, followed by saturated
NaCl solution, dried over anhydrous sodium sulfate, filtered
and evaporated. The residue was purified by silica gel
column chromatography (eluted with 5:100:1 MeOH/CH2Cl2/TEA) to
afford the product.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-
      ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-
      diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-
N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) were added to
a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-
dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol)
dissolved in CH2Cl2 (20 mL) under an atmosphere of argon. The
reaction mixture was stirred overnight and the solvent
evaporated. The resulting residue was purified by silica gel
column chromatography with EtOAc as the eluent to afford the
title compound.

Example 2
Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

5   Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages.

10 The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1 M NH$_4$oAc

15 solution.   Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by

20 reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as

25 described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499,

30 respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

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Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as
5   described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

10   Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethyl-hydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked
15 oligonucleosides, and methyleneaminocarbonyl linked oligo-nucleosides, also identified as amide-4 linked oligonucleo-sides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023,
20 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

        Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

25   Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

30 **PNA Synthesis**

        Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential

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Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-
23. They may also be prepared in accordance with U.S.
Patents 5,539,082, 5,700,922, and 5,719,262, herein
incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed
oligonucleotides/oligonucleosides of the invention can be of
several different types. These include a first type wherein
the "gap" segment of linked nucleosides is positioned between
5' and 3' "wing" segments of linked nucleosides and a second
"open end" type wherein the "gap" segment is located at
either the 3' or the 5' terminus of the oligomeric compound.
Oligonucleotides of the first type are also known in the art
as "gapmers" or gapped oligonucleotides. Oligonucleotides of
the second type are also known in the art as "hemimers" or
"wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric
Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl
phosphorothioate and 2'-deoxy phosphorothioate oligo-
nucleotide segments are synthesized using an Applied
Biosystems automated DNA synthesizer Model 394, as above.
Oligonucleotides are synthesized using the automated
synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-
amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-
methyl-3'-O-phosphoramidite for 5' and 3' wings. The
standard synthesis cycle is modified by incorporating
coupling steps with increased reaction times for the 5'-
dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully
protected oligonucleotide is cleaved from the support and
deprotected in concentrated ammonia (NH4OH) for 12-16 hr at

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55°C.   The deprotected oligo is then recovered by an
appropriate method (precipitation, column chromatography,
volume reduced *in vacuo* and analyzed spetrophotometrically
for yield and for purity by capillary electrophoresis and by
5   mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-
(Methoxyethyl)] Chimeric Phosphorothioate
Oligonucleotides**

10   [2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-
(methoxyethyl)] chimeric phosphorothioate oligonucleotides
were prepared as per the procedure above for the 2'-O-methyl
chimeric oligonucleotide, with the substitution of 2'-O-
(methoxyethyl) amidites for the 2'-O-methyl amidites.

15

**[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy
Phosphorothioate]--[2'-O-(2-Methoxyethyl)
Phosphodiester] Chimeric Oligonucleotides**

   [2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy
20   phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester]
chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with
the substitution of 2'-O-(methoxyethyl) amidites for the 2'-
O-methyl amidites, oxidation with iodine to generate the
25   phosphodiester internucleotide linkages within the wing
portions of the chimeric structures and sulfurization
utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage
Reagent) to generate the phosphorothioate internucleotide
linkages for the center gap.

30   Other chimeric oligonucleotides, chimeric
oligonucleosides and mixed chimeric
oligonucleotides/oligonucleosides are synthesized according
to United States patent 5,623,065, herein incorporated by

reference.

Example 6

Oligonucleotide Isolation

5   After cleavage from the controlled pore glass solid
support and deblocking in concentrated ammonium hydroxide at
55°C for 12-16 hours, the oligonucleotides or oligonucleosides
are recovered by precipitation out of 1 M NH_4OAc with >3
volumes of ethanol. Synthesized oligonucleotides were
10   analyzed by electrospray mass spectroscopy (molecular weight
determination) and by capillary gel electrophoresis and
judged to be at least 70% full length material. The relative
amounts of phosphorothioate and phosphodiester linkages
obtained in the synthesis was determined by the ratio of
15   correct molecular weight relative to the -16 amu product (+/-
32 +/-48). For some studies oligonucleotides were purified
by HPLC, as described by Chiang et al., J. Biol. Chem. **1991**,
266, 18162-18171. Results obtained with HPLC-purified
material were similar to those obtained with non-HPLC
20   purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III)
25   phosphoramidite chemistry on an automated synthesizer capable
of assembling 96 sequences simultaneously in a 96-well
format. Phosphodiester internucleotide linkages were
afforded by oxidation with aqueous iodine. Phosphorothioate
internucleotide linkages were generated by sulfurization
30   utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage
Reagent) in anhydrous acetonitrile. Standard base-protected
beta-cyanoethyl-diiso-propyl phosphoramidites were purchased
from commercial vendors (e.g. PE-Applied Biosystems, Foster

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City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

5　　　Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and
10　test plate samples are then diluted utilizing robotic pipettors.

Example 8

15　**Oligonucleotide Analysis - 96-Well Plate Format**
　　　The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in
20　either the 96-well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test
25　plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

30　**Example 9**
　　Cell culture and oligonucleotide treatment
　　　The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types

provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following cell types are provided for illustrative purposes, but other

5 cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, ribonuclease protection assays, or RT-PCR.

10

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in

15 complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by

20 trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates

25 and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained

30 from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA),

penicillin 100 units per mL, and streptomycin 100 micrograms
per mL (Invitrogen Corporation, Carlsbad, CA). Cells were
routinely passaged by trypsinization and dilution when they
reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained
from the Clonetics Corporation (Walkersville, MD). NHDFs
were routinely maintained in Fibroblast Growth Medium
(Clonetics Corporation, Walkersville, MD) supplemented as
recommended by the supplier. Cells were maintained for up to
10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from
the Clonetics Corporation (Walkersville, MD). HEKs were
routinely maintained in Keratinocyte Growth Medium (Clonetics
Corporation, Walkersville, MD) formulated as recommended by
the supplier. Cells were routinely maintained for up to 10
passages as recommended by the supplier.

Treatment with antisense compounds:

When cells reached 70% confluency, they were treated
with oligonucleotide. For cells grown in 96-well plates,
wells were washed once with 100 μL OPTI-MEM™-1 reduced-serum
medium (Invitrogen Corporation, Carlsbad, CA) and then
treated with 130 μL of OPTI-MEM™-1 containing 3.75 μg/mL
LIPOFECTIN™ (Invitrogen Corporation, Carlsbad, CA) and the
desired concentration of oligonucleotide. After 4-7 hours of
treatment, the medium was replaced with fresh medium. Cells
were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from
cell line to cell line. To determine the optimal

oligonucleotide concentration for a particular cell line, the
cells are treated with a positive control oligonucleotide at
a range of concentrations. For human cells the positive
control oligonucleotide is selected from either ISIS 13920

5   (**TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1) which is targeted to
human H-ras, or ISIS 18078, (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO:
2) which is targeted to human Jun-N-terminal kinase-2 (JNK2).
Both controls are 2'-O-methoxyethyl gapmers (2'-O-
methoxyethyls shown in bold) with a phosphorothioate

10   backbone. For mouse or rat cells the positive control
oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID
NO: 3, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown
in bold) with a phosphorothioate backbone which is targeted
to both mouse and rat c-raf. The concentration of positive

15   control oligonucleotide that results in 80% inhibition of c-
Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is
then utilized as the screening concentration for new
oligonucleotides in subsequent experiments for that cell
line. If 80% inhibition is not achieved, the lowest

20   concentration of positive control oligonucleotide that
results in 60% inhibition of H-ras or c-raf mRNA is then
utilized as the oligonucleotide screening concentration in
subsequent experiments for that cell line. If 60% inhibition
is not achieved, that particular cell line is deemed as

25   unsuitable for oligonucleotide transfection experiments. The
concentrations of antisense oligonucleotides used herein are
from 50 nM to 300 nM.

Example 10

30   **Analysis of oligonucleotide inhibition of IL-1 receptor-
associated kinase-1 expression**

        Antisense modulation of IL-1 receptor-associated kinase-
1 expression can be assayed in a variety of ways known in the

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art. For example, IL-1 receptor-associated kinase-1 mRNA
levels can be quantitated by, e.g., Northern blot analysis,
competitive polymerase chain reaction (PCR), or real-time PCR
(RT-PCR). Real-time quantitative PCR is presently preferred.

5   RNA analysis can be performed on total cellular RNA or
poly(A)+ mRNA. The preferred method of RNA analysis of the
present invention is the use of total cellular RNA as
described in other examples herein. Methods of RNA isolation
are taught in, for example, Ausubel, F.*M. et al.*, *Current*

10   *Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and
4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot
analysis is routine in the art and is taught in, for example,
Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*,
Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**.

15   Real-time quantitative (PCR) can be conveniently accomplished
using the commercially available ABI PRISM™ 7700 Sequence
Detection System, available from PE-Applied Biosystems,
Foster City, CA and used according to manufacturer's
instructions.

20   Protein levels of IL-1 receptor-associated kinase-1 can
be quantitated in a variety of ways well known in the art,
such as immunoprecipitation, Western blot analysis
(immunoblotting), ELISA or fluorescence-activated cell
sorting (FACS). Antibodies directed to IL-1 receptor-

25   associated kinase-1 can be identified and obtained from a
variety of sources, such as the MSRS catalog of antibodies
(Aerie Corporation, Birmingham, MI), or can be prepared via
conventional antibody generation methods. Methods for
preparation of polyclonal antisera are taught in, for

30   example, Ausubel, F.M. *et al.*, (*Current Protocols in
Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley
& Sons, Inc., **1997**). Preparation of monoclonal antibodies is
taught in, for example, Ausubel, F.M. *et al.*, (*Current*

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Protocols in Molecular Biology, Volume 2, pp. 11.4.1-11.11.5,
John Wiley & Sons, Inc., **1997**).

        Immunoprecipitation methods are standard in the art and
can be found at, for example, Ausubel, F.M. *et al.*, (*Current*
5   *Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-
10.16.11, John Wiley & Sons, Inc., **1998**). Western blot
(immunoblot) analysis is standard in the art and can be found
at, for example, Ausubel, F.M. *et al.*, (*Current Protocols in
Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley &
10 Sons, Inc., **1997**). Enzyme-linked immunosorbent assays
(ELISA) are standard in the art and can be found at, for
example, Ausubel, F.M. *et al.*, (*Current Protocols in
Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley &
Sons, Inc., **1991**).

15

Example 11
Poly(A)+ mRNA isolation

        Poly(A)+ mRNA was isolated according to Miura *et al.*,
(*Clin. Chem.*, **1996**, *42*, 1758-1764). Other methods for
20 poly(A)+ mRNA isolation are taught in, for example, Ausubel,
F.M. *et al.*, (*Current Protocols in Molecular Biology*, Volume
1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**). Briefly,
for cells grown on 96-well plates, growth medium was removed
from the cells and each well was washed with 200 µL cold PBS.
25 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M
NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was
added to each well, the plate was gently agitated and then
incubated at room temperature for five minutes. 55 µL of
lysate was transferred to Oligo d(T) coated 96-well plates
30 (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes
at room temperature, washed 3 times with 200 µL of wash
buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After
the final wash, the plate was blotted on paper towels to

remove excess wash buffer and then air-dried for 5 minutes.
60 μL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to
70°C, was added to each well, the plate was incubated on a
90°C hot plate for 5 minutes, and the eluate was then
5   transferred to a fresh 96-well plate.

       Cells grown on 100 mm or other standard plates may be
treated similarly, using appropriate volumes of all
solutions.

10   **Example 12**

Total RNA Isolation

       Total RNA was isolated using an RNEASY 96™ kit and
buffers purchased from Qiagen Inc. (Valencia, CA) following
the manufacturer's recommended procedures. Briefly, for
15   cells grown on 96-well plates, growth medium was removed from
the cells and each well was washed with 200 μL cold PBS. 150
μL Buffer RLT was added to each well and the plate vigorously
agitated for 20 seconds. 150 μL of 70% ethanol was then
added to each well and the contents mixed by pipetting three
20   times up and down. The samples were then transferred to the
RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted
with a waste collection tray and attached to a vacuum source.
Vacuum was applied for 1 minute. 500 μL of Buffer RW1 was
added to each well of the RNEASY 96™ plate and incubated for
25   15 minutes and the vacuum was again applied for 1 minute. An
additional 500 μL of Buffer RW1 was added to each well of the
RNEASY 96™ plate and the vacuum was applied for 2 minutes. 1
mL of Buffer RPE was then added to each well of the RNEASY
96™ plate and the vacuum applied for a period of 90 seconds.
30   The Buffer RPE wash was then repeated and the vacuum was
applied for an additional 3 minutes. The plate was then
removed from the QIAVAC™ manifold and blotted dry on paper

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towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 170 μL water into each well, incubating 1 minute, and then applying

5   the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck

10   where the pipetting, DNase treatment and elution steps are carried out.

Example 13

15   **Real-time Quantitative PCR Analysis of IL-1 receptor-associated kinase-1 mRNA Levels**

Quantitation of IL-1 receptor-associated kinase-1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied

20   Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification

25   products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two

30   fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe

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and a quencher dye (e.g., TAMRA, obtained from either PE-
Applied Biosystems, Foster City, CA, Operon Technologies
Inc., Alameda, CA or Integrated DNA Technologies Inc.,
Coralville, IA) is attached to the 3' end of the probe. When
the probe and dyes are intact, reporter dye emission is
quenched by the proximity of the 3' quencher dye. During
amplification, annealing of the probe to the target sequence
creates a substrate that can be cleaved by the 5'-exonuclease
activity of Taq polymerase. During the extension phase of
the PCR amplification cycle, cleavage of the probe by Taq
polymerase releases the reporter dye from the remainder of
the probe (and hence from the quencher moiety) and a
sequence-specific fluorescent signal is generated. With each
cycle, additional reporter dye molecules are cleaved from
their respective probes, and the fluorescence intensity is
monitored at regular intervals by laser optics built into the
ABI PRISM™ 7700 Sequence Detection System. In each assay, a
series of parallel reactions containing serial dilutions of
mRNA from untreated control samples generates a standard
curve that is used to quantitate the percent inhibition after
antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets
specific to the target gene being measured are evaluated for
their ability to be "multiplexed" with a GAPDH amplification
reaction. In multiplexing, both the target gene and the
internal standard gene GAPDH are amplified concurrently in a
single sample. In this analysis, mRNA isolated from
untreated cells is serially diluted. Each dilution is
amplified in the presence of primer-probe sets specific for
GAPDH only, target gene only ("single-plexing"), or both
(multiplexing). Following PCR amplification, standard curves
of GAPDH and target mRNA signal as a function of dilution are
generated from both the single-plexed and multiplexed

samples. If both the slope and correlation coefficient of
the GAPDH and target signals generated from the multiplexed
samples fall within 10% of their corresponding values
generated from the single-plexed samples, the primer-probe
5   set specific for that target is deemed multiplexable. Other
methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen Corporation,
(Carlsbad, CA). RT-PCR reactions were carried out by adding
20 µL PCR cocktail (2.5x PCR buffer (-MgCl2), 6.6 mM MgCl2,
10   375 µM each of dATP, dCTP, dCTP and dGTP, 375 nM each of
forward primer and reverse primer, 125 nM of probe, 4 Units
RNAse inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV
reverse transcriptase, and 2.5x ROX dye) to 96-well plates
containing 30 µL total RNA solution. The RT reaction was
15   carried out by incubation for 30 minutes at 48°C. Following a
10 minute incubation at 95°C to activate the PLATINUM® Taq, 40
cycles of a two-step PCR protocol were carried out: 95°C for
15 seconds (denaturation) followed by 60°C for 1.5 minutes
(annealing/extension).

20   Gene target quantities obtained by real time RT-PCR are
normalized using either the expression level of GAPDH, a gene
whose expression is constant, or by quantifying total RNA
using RiboGreenTM (Molecular Probes, Inc. Eugene, OR). GAPDH
expression is quantified by real time RT-PCR, by being run
25   simultaneously with the target, multiplexing, or separately.
Total RNA is quantified using RiboGreenTM RNA quantification
reagent from Molecular Probes. Methods of RNA quantification
by RiboGreenTM are taught in Jones, L.J., et al, (Analytical
Biochemistry, 1998, 265, 368-374).

30   In this assay, 170 µL of RiboGreenTM working reagent
(RiboGreenTM reagent diluted 1:350 in 10mM Tris-HCl, 1 mM
EDTA, pH 7.5) is pipetted into a 96-well plate containing 30

μL purified, cellular RNA. The plate is read in a CytoFluor
4000 (PE Applied Biosystems) with excitation at 480nm and
emission at 520nm.

     Probes and primers to human IL-1 receptor-associated
5   kinase-1 were designed to hybridize to a human IL-1 receptor-
associated kinase-1 sequence, using published sequence
information (GenBank accession number L76191.1, incorporated
herein as SEQ ID NO:4). For human IL-1 receptor-associated
kinase-1 the PCR primers were:

10 forward primer: ACTTCTCGGAGGAGCTCAAGATC (SEQ ID NO: 5)
reverse primer: GCATACACCGTGTTCCTCATCA (SEQ ID NO: 6) and the
PCR probe was: FAM-CGCCCGGTACACGCACCCAA-TAMRA
(SEQ ID NO: 7) where FAM is the fluorescent dye and TAMRA is
the quencher dye. For human GAPDH the PCR primers were:

15 forward primer: GAAGGTGAAGGTCGGAGTC(SEQ ID NO:8)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:9) and the
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID
NO: 10) where JOE is the fluorescent reporter dye and TAMRA
is the quencher dye.

20

Example 14
Northern blot analysis of IL-1 receptor-associated kinase-1
mRNA levels

25   Eighteen hours after antisense treatment, cell
monolayers were washed twice with cold PBS and lysed in 1 mL
RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was
prepared following manufacturer's recommended protocols.
Twenty micrograms of total RNA was fractionated by
30 electrophoresis through 1.2% agarose gels containing 1.1%
formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon,
OH). RNA was transferred from the gel to HYBOND™-N+ nylon
membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by

overnight capillary transfer using a Northern/Southern

Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX).

RNA transfer was confirmed by UV visualization. Membranes

were fixed by UV cross-linking using a STRATALINKER™ UV

5   Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then

probed using QUICKHYB™ hybridization solution (Stratagene, La

Jolla, CA) using manufacturer's recommendations for stringent

conditions.

       To detect human IL-1 receptor-associated kinase-1, a

10   human IL-1 receptor-associated kinase-1 specific probe was

prepared by PCR using the forward primer

ACTTCTCGGAGGAGCTCAAGATC (SEQ ID NO: 5) and the reverse primer

GCATACACCGTGTTCCTCATCA (SEQ ID NO: 6). To normalize for

variations in loading and transfer efficiency membranes were

15   stripped and probed for human glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

       Hybridized membranes were visualized and quantitated

using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3

(Molecular Dynamics, Sunnyvale, CA). Data was normalized to

20   GAPDH levels in untreated controls.

       Example 15

       Antisense inhibition of human IL-1 receptor-associated

25   **kinase-1 expression by chimeric phosphorothioate**

       oligonucleotides having 2'-MOE wings and a deoxy gap

       In accordance with the present invention, a series of

oligonucleotides were designed to target different regions of

the human IL-1 receptor-associated kinase-1 RNA, using

30   published sequences (GenBank accession number L76191.1,

incorporated herein as SEQ ID NO: 4, nucleotides 1 to 13000

of GenBank accession number AF031075.1, the complement of

which is incorporated herein as SEQ ID NO: 11, GenBank

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accession number BG479917.1, incorporated herein as SEQ ID
NO: 12, and GenBank accession number AL581159.1, the
complement of which is incorporated herein as SEQ ID NO: 13).
The oligonucleotides are shown in Table 1. "Target site"
5   indicates the first (5'-most) nucleotide number on the
particular target sequence to which the oligonucleotide
binds. All compounds in Table 1 are chimeric
oligonucleotides ("gapmers") 20 nucleotides in length,
composed of a central "gap" region consisting of ten 2'-
10   deoxynucleotides, which is flanked on both sides (5' and 3'
directions) by five-nucleotide "wings". The wings are
composed of 2'-methoxyethyl (2'-MOE) nucleotides. The
internucleoside (backbone) linkages are phosphorothioate
(P=S) throughout the oligonucleotide. All cytidine residues
15   are 5-methylcytidines. The compounds were analyzed for their
effect on human IL-1 receptor-associated kinase-1 mRNA levels
by quantitative real-time PCR as described in other examples
herein. Data are averages from two experiments in which T-24
cells were treated with the antisense oligonucleotides of the
20   present invention. The positive control for each datapoint
is identified in the table by sequence ID number. If
present, "N.D." indicates "no data".

Table 1

25   **Inhibition of human IL-1 receptor-associated kinase-1 mRNA
levels by chimeric phosphorothioate oligonucleotides having
2'-MOE wings and a deoxy gap**

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
151391	3'UTR	4	3139	cctggcttgcaggccaccac	91	14	2
151392	3'UTR	4	2424	gatgccagccttccttgccc	94	15	2
151393	Coding	4	965	cagtggagacggtcctccag	90	16	2
151394	3'UTR	4	3196	cttgtggcctccgaagcctg	96	17	2
151395	Coding	4	2127	ggacgacagcagctgcaggc	84	18	2
151396	Coding	4	1448	tctgcagccagacctgcttg	60	19	2

151397	Coding	4	1443	agccagacctgcttgcagtg	92	20	2
151398	3'UTR	4	3450	gtgaagcctgtgctacagcc	93	21	2
151399	Coding	4	191	tggcaccagtcggcgggctc	92	22	2
151400	Coding	4	2070	gaccatcttctgtcgggcag	81	23	2
151401	Coding	4	1264	ccagccttcccgtcttgatg	92	24	2
151402	3'UTR	4	2465	agccagcagcctcccaacat	94	25	2
151403	Stop Codon	4	2199	tcagctctgaaattcatcac	94	26	2
151404	Coding	4	1995	cagtccttccacggctgtgg	90	27	2
151405	Coding	4	1315	ccaaggtctctagcactacc	95	28	2
151406	3'UTR	4	3269	ctaggtcttgggcctagcta	85	29	2
151407	Coding	4	766	acaccgtgttcctcatcacc	99	30	2
151408	Coding	4	2195	ctctgaaattcatcactttc	32	31	2
151409	Coding	4	1598	tgggtcataggaggcctcct	64	32	2
151410	3'UTR	4	3171	gctcggagctcgtctgtggc	11	33	2
151411	Coding	4	1348	tggcaccgtgcgtcttcaca	91	34	2
151412	Coding	4	146	cggcacatgacccagggcgg	94	35	2
151413	Coding	4	1322	tgaccagccaaggtctctag	0	36	2
151414	Coding	4	1383	ctcctcttccaccaggtctt	89	37	2
151415	3'UTR	4	2252	tgaccatgagaactttgact	97	38	2
151416	Coding	4	2001	aagggccagtccttccacgg	1	39	2
151417	Coding	4	1709	ctggacacgtaggagttctc	96	40	2
151418	Coding	4	1711	tgctggacacgtaggagttc	90	41	2
151419	Coding	4	2069	accatcttctgtcgggcagg	92	42	2
151420	Coding	4	1432	cttgcagtgtgctctgggtg	78	43	2
151421	Coding	4	854	ctggacagctgctccacctc	93	44	2
151422	3'UTR	4	3436	acagccctgggctacttttg	89	45	2
151423	3'UTR	4	2390	ggaggcagggttccactctg	90	46	2
151424	Coding	4	1446	tgcagccagacctgcttgca	71	47	2
151425	3'UTR	4	3246	cttgggtagtggcccctctg	94	48	2
151426	Coding	4	1998	ggccagtccttccacggctg	92	49	2
151427	Coding	4	901	tctgagcacagtagccagca	97	50	2
194303	Coding	4	341	cggagcagctgcaggtgcgt	79	51	2
194304	Coding	4	556	aagcagggcttggaaccagg	84	52	2
194305	Coding	4	715	cgatcttgagctcctccgag	81	53	2
194306	Coding	4	840	cacctcggtcaggaagctct	87	54	2
194307	Coding	4	933	caggaagccgtacaccaggc	88	55	2
194308	Coding	4	1211	actgtctgtgtccgggccac	76	56	2
194309	Coding	4	1548	cagctggcccaggcccaggc	50	57	2
194310	Coding	4	1574	gcccggcggtgcaggcagca	87	58	2
194311	Coding	4	1621	gcttctctagcctctcgtac	81	59	2
194312	Coding	4	1748	ggctgccatggagcagcccc	23	60	2
194313	Coding	4	1795	gcagctgctctgctgcctgg	64	61	2
194314	Coding	4	1848	agagaggccgcctaggctct	76	62	2
194315	Coding	4	1890	cagagggcagcttggagtca	43	63	2
194316	Coding	4	2084	agggccagcttctggaccat	87	64	2
194317	Stop Codon	4	2209	ggtgaacacatcagctctga	0	65	2
194318	3'UTR	4	2577	agctgctgccagaggcctgg	81	66	2
194319	3'UTR	4	2994	ttacagccatacttcacttt	75	67	2
194320	3'UTR	4	3029	aattctcgcttcttgctagg	89	68	2
194321	3'UTR	4	3115	ggccagctcgcaggtcccca	91	69	2
194322	3'UTR	4	3332	ccttccctgtctgccatgct	90	70	2
194323	3'UTR	4	3413	acgcaagaggacactcggtt	92	71	2
194324	3'UTR	4	3469	ggctgaacacaaaatcactg	88	72	2
194325	3'UTR	4	3477	tgactcacggctgaacacaa	85	73	2
194326	3'UTR	4	3544	atacgttttattactcaag	89	74	2

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194327	3'UTR	4	3551	agggaacatacgtttttatt	30.	75	2
194328	intron	11	2441	cctggaaaagcttcataaag	32	76	2
194329	exon	11	3229	ctggcctcacctggacagct	42	77	2
194330	intron	11	4066	agaccctccagctacgctgc	36	78	2
194331	intron	11	5276	ggagagcccacttgaagaca	61	79	2
194332	exon	11	7478	agctggctacctgggtcata	37	80	2
194333	intron	11	7534	tacagagcaaggcctggaat	62	81	2
194334	intron	11	7694	tccttctctctatgtgaagg	62	82	2
194335	intron	11	9602	aggcccaagcctacagaagg	34	83	2
194336	genomic	12	184	aggccagctggcccaggcgc	19	84	2
194337	3' UTR	13	136	acccaggctggagatggcgg	61	85	2

As shown in Table 1, SEQ ID NOs 14, 15, 16, 17, 18, 19,
20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 34, 35, 37,
38, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53,
54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72,
73, 74, 79, 81, 82 and 85 demonstrated at least 60%
inhibition of human IL-1 receptor-associated kinase-1
expression in this assay and are therefore preferred. The
target sites to which these preferred sequences are
complementary are herein referred to as "preferred target
regions" and are therefore preferred sites for targeting by
compounds of the present invention. These preferred target
regions are shown in Table 2. The sequences represent the
reverse complement of the preferred antisense compounds shown
in Table 1. "Target site" indicates the first (5'-most)
nucleotide number of the corresponding target nucleic acid.
Also shown in Table 2 is the species in which each of the
preferred target regions was found.

Table 2
Sequence and position of preferred target regions identified
in IL-1 receptor-associated kinase-1.

SITE ID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
66912	4	3139	gtggtggcctgcaagccagg	14	*H. sapiens*	86

66913	4	2424	gggcaaggaaggctggcatc	15	H. sapiens	87
66914	4	965	ctggaggaccgtctccactg	16	H. sapiens	88
66915	4	3196	caggcttcggaggccacaag	17	H. sapiens	89
66916	4	2127	gcctgcagctgctgtcgtcc	18	H. sapiens	90
66917	4	1448	caagcaggtctggctgcaga	19	H. sapiens	91
66918	4	1443	cactgcaagcaggtctggct	20	H. sapiens	92
66919	4	3450	ggctgtagcacaggcttcac	21	H. sapiens	93
66920	4	191	gagcccgccgactggtgcca	22	H. sapiens	94
66921	4	2070	ctgcccgacagaagatggtc	23	H. sapiens	95
66922	4	1264	catcaagacgggaaggctgg	24	H. sapiens	96
66923	4	2465	atgttgggaggctgctggct	25	H. sapiens	97
66924	4	2199	gtgatgaatttcagagctga	26	H. sapiens	98
66925	4	1995	ccacagccgtggaaggactg	27	H. sapiens	99
66926	4	1315	ggtagtgctagagaccttgg	28	H. sapiens	100
66927	4	3269	tagctaggcccaagacctag	29	H. sapiens	101
66928	4	766	ggtgatgaggaacacggtgt	30	H. sapiens	102
66930	4	1598	aggaggcctcctatgaccca	32	H. sapiens	103
66932	4	1348	tgtgaagacgcacggtgcca	34	H. sapiens	104
66933	4	146	ccgccctgggtcatgtgccg	35	H. sapiens	105
66935	4	1383	aagacctggtggaagaggag	37	H. sapiens	106
66936	4	2252	agtcaaagttctcatggtca	38	H. sapiens	107
66938	4	1709	gagaactcctacgtgtccag	40	H. sapiens	108
66939	4	1711	gaactcctacgtgtccagca	41	H. sapiens	109
66940	4	2069	cctgcccgacagaagatggt	42	H. sapiens	110
66941	4	1432	cacccagagcacactgcaag	43	H. sapiens	111
66942	4	854	gaggtggagcagctgtccag	44	H. sapiens	112
66943	4	3436	caaaagtagcccagggctgt	45	H. sapiens	113
66944	4	2390	cagagtggaaccctgcctcc	46	H. sapiens	114
66945	4	1446	tgcaagcaggtctggctgca	47	H. sapiens	115
66946	4	3246	cagaggggccactacccaag	48	H. sapiens	116
66947	4	1998	cagccgtggaaggactggcc	49	H. sapiens	117
66948	4	901	tgctggctactgtgctcaga	50	H. sapiens	118
112415	4	341	acgcacctgcagctgctccg	51	H. sapiens	119
112416	4	556	cctggttccaagccctgctt	52	H. sapiens	120
112417	4	715	ctcggaggagctcaagatcg	53	H. sapiens	121
112418	4	840	agagcttcctgaccgaggtg	54	H. sapiens	122
112419	4	933	gcctggtgtacggcttcctg	55	H. sapiens	123
112420	4	1211	gtggcccggacacagacagt	56	H. sapiens	124
112422	4	1574	tgctgcctgcaccgccgggc	58	H. sapiens	125
112423	4	1621	gtacgagaggctagagaagc	59	H. sapiens	126
112425	4	1795	ccaggcagcagagcagctgc	61	H. sapiens	127
112426	4	1848	agagcctaggcggcctctct	62	H. sapiens	128
112428	4	2084	atggtccagaagctggccct	64	H. sapiens	129
112430	4	2577	ccaggcctctggcagcagct	66	H. sapiens	130
112431	4	2994	aaagtgaagtatggctgtaa	67	H. sapiens	131
112432	4	3029	cctagcaagaagcgagaatt	68	H. sapiens	132
112433	4	3115	tggggacctgcgagctggcc	69	H. sapiens	133
112434	4	3332	agcatggcagacagggaagg	70	H. sapiens	134
112435	4	3413	aaccgagtgtcctcttgcgt	71	H. sapiens	135
112436	4	3469	cagtgattttgtgttcagcc	72	H. sapiens	136
112437	4	3477	ttgtgttcagccgtgagtca	73	H. sapiens	137
112438	4	3544	cttgagtaataaaaacgtat	74	H. sapiens	138
112443	11	5276	tgtcttcaagtgggctctcc	79	H. sapiens	139
112445	11	7534	attccaggccttgctctgta	81	H. sapiens	140
112446	11	7694	ccttcacatagagagaagga	82	H. sapiens	141
112449	13	136	ccgccatctccagcctgggt	85	H. sapiens	142

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As these "preferred target regions" have been found by
experimentation to be open to, and accessible for,
5   hybridization with the antisense compounds of the present
invention, one of skill in the art will recognize or be able
to ascertain, using no more than routine experimentation,
further embodiments of the invention that encompass other
compounds that specifically hybridize to these sites and
10 consequently inhibit the expression of IL-1 receptor-
associated kinase-1.

Example 16
15 **Western blot analysis of IL-1 receptor-associated kinase-1
protein levels**
Western blot analysis (immunoblot analysis) is carried
out using standard methods. Cells are harvested 16-20 h
after oligonucleotide treatment, washed once with PBS,
20 suspended in Laemmli buffer (100 ul/well), boiled for 5
minutes and loaded on a 16% SDS-PAGE gel. Gels are run for
1.5 hours at 150 V, and transferred to membrane for western
blotting. Appropriate primary antibody directed to IL-1
receptor-associated kinase-1 is used, with a radiolabeled or
25 fluorescently labeled secondary antibody directed against the
primary antibody species. Bands are visualized using a
PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).